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Identification chimique de métabolites secondaires de certains microorganismes, évaluation de leur effet dans les domaines pharmaceutiques et agronomiques

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Liste des Abréviations

ACh	acétylthiocholine iodide
AChE	acétylcholinestérase
AcOEt (EtOAc)	acétate d'éthyle
BuOH	n-butanol
CCM	chromatographie sur Couche Mince
GC	chromatographie en Phase Gazeuse
GC-MS)	chromatographie en Phase Gazeuse couplée à la Spectrométrie de Masse
Cyclo	cyclohexane
d	doublet
DCM	dichlorométhane
dd	doublet dédoublé
DKP	dicétopipérazine
DMSO	diméthylsulfoxyde
EP	éther de pétrole
éq (equi)	équivalent
HRSM	Spectrométrie de masse à Haute Résolution
g	gramme
h	heure
HCl	acide chlorhydrique
Hz	Hertz
IR	Infra rouge
IP	pourcentage d'inhibition
J	constante de couplage exprimée en Hz
kg	kilogramme
q	quadruplet
m	multiplet
MeOH	méthanol
mg	milligramme
min	minute
mL	millilitre
mM	milli Molaire

MTT	Bromure de 3-(4,5-dimethylthiazol-2-yl)-2,5-diphényl tétrazolium)
m/z	rapport masse/charge électrique
NIST	National Institute of Standards and Technology
nm	nanomètre
n.r.	nombre
ppm	partie par million
Rdt	rendement
RMN ^{13}C	résonnance Magnétique Nucléaire du carbone 13
RMN ^{1}H	résonnance Magnétique Nucléaire du proton
r.t.	temps de rétention
s	singulet
T	Température
t	triplet
THF	tetrahydrofurane
UV	Ultraviolet
V/V	volume/volume
δ	déplacement chimique exprimé en ppm
°C	degré Celsius
µg	Micro-gramme
µL	Micro-litre
µM	Micro-molaire

Introduction générale

Depuis l'antiquité, la nature, et par son impressionnante richesse, son biodiversité, sa complexité et en plus de sa beauté bien souvent, attire l'attention des chimistes en les offrant une multitude des molécules ayant des propriétés biologiques intéressantes : thérapeutiques, agronomique, agroalimentaire. Aujourd'hui, les êtres humains se servent de la nature (plante, microorganismes, organismes marins…) pour identifier et isoler des principes actifs tels que les alcaloïdes, les antifongiques, les antibiotiques et qui sont doués de plusieurs activités importantes.

En plus des plantes qui ont toujours eu une place importante dans l'arsenal thérapeutique de l'humanité, les microorganismes (levures, bactéries, moisissures et infusoires) occupent une place importante dans la vie quotidienne de l'être humain (utilisation thérapeutique, fabrication des produits alimentaires fermentés…). Ces microorganismes, ubiquistes dans notre vie (air, sol, eau, peau...) ne cessent d'occuper une place de plus en plus importante dans notre vie et sont à l'origine de nombreux principes actifs tels les antibiotiques : les bêta-lactamines (les pénicillines), les aminosides, les sulfamides, les dicétopipérazines, etc., les alcools, les cétones, les aldéhydes, les acides carboxyliques, les composés soufrés, etc.

La nature présente une source d'inspiration pour les chercheurs, et conscients de ce fait, plusieurs équipes de recherches dans le monde ont focalisé leurs travaux de recherche espérant exploiter la nature pour fournir des substances naturelles aux propriétés biologiques intéressantes.

La présente thèse intitulé « **Identification chimique de métabolites secondaires de certains microorganismes, évaluation de leurs effets dans les domaines pharmaceutiques et agronomiques** » et qui s'inscrit dans le cadre de la valorisation du dit programme a été réalisée en co-tutelle sous la direction de Mr **Jalloul BOUAJILA** Maître de conférences HDR à la Faculté de pharmacie de Toulouse, Université Paul Sabatier (France) et Mr **Hichem BEN JANNET** Professeur à la Faculté des Sciences de l'Université de Monastir (Tunisie).

Les travaux de recherche de la présente thèse, qui constitue une autre contribution aux efforts de notre équipe de recherche à valoriser les ressources naturelles, s'inscrivent dans le cadre de la recherche de molécules bioactives à partir de microorganismes pour des applications pharmaceutiques et agronomiques.

Durant les travaux de cette thèse, nous nous sommes intéressés à l'étude de deux souches bactériennes isolés et identifiés par nos co-équipiers (Agronutrition) : *Burkholderia* sp. AGN02 et *Bacillus megaterium* AGN01.

Le travail présenté dans ce mémoire s'articule en quatre parties :

- La première partie est consacrée à une synthèse bibliographique dans laquelle on décrit les bactéries étudiées ainsi que les métabolites secondaires principaux identifiés dans ces deux genres.
- La deuxième partie décrit l'étude de l'influence de la nature de source du carbone, utilisé lors de la cultivation de *Burkholderia* sp. AGN02, sur la production des métabolites volatils. De plus, notre intérêt s'est porté sur l'évaluation biologique des substances naturelles extraites de *Burkholderia* sp. AGN02, en s'appuyant sur l'influence de la nature de source de carbone sur les essais biologiques.
- La troisième partie a été consacrée à l'investigation de l'impact des interactions bactérie-bactérie sur la composition chimique (composés phénoliques, HPLC et GC/HRMS) ainsi que sur les résultats des activités biologiques des différents extraits préparés à partir de *Bacillus megaterium* AGN01, *Burkholderia* sp. AGN02 et leur mélange.
- Nous avons consacré le quatrième chapitre à l'étude de l'effet de stockage sur la composition chimique de bactéries étudiées. L'extraction, l'identification des molécules volatiles à partir de *Bacillus megaterium* AGN01, *Burkholderia* sp. AGN02 et leur mélange ainsi que l'évaluation biologique des différents extraits préparés ont été faits.
- La cinquième partie décrit la synthèse d'analogues structuraux type dicétopipérazine ainsi que l'évaluation des activités biologiques des composés synthétisés, suivi d'une conclusion générale vient pour clôturer ce travail, qui résume nos résultats et qui propose des perspectives pour des études ultérieures.

Chapitre I : Etude Bibliographique

1. Production des métabolites secondaires

La composition chimique des métabolites secondaires émis par les microorganismes est complexe et est constituée de deux grandes fractions. La première fraction est dite non volatile, composés organiques non volatils microbiens (CONVM) est composé par une large variété de composés appartenant à plusieurs classes de familles chimiques à savoir, les macrolides, les aminoglycosides, les β-lactamines, les quinolines, les sulfamides, les quinolones, les peptides cycliques tels que les dérivés de dicétopipérazines, etc ayant des valeurs ajoutées et jouant un rôle fondamental dans l'activité biologique du microorganisme.
La deuxième fraction dite volatile, composés organiques volatils, est produite par une grande variété des microorganismes allant des bactéries aux champignons. Les composés organiques volatils microbiens (COVM) se produisent généralement en tant qu'un mélange complexe de composés lipophiles ayant une basse masse moléculaire provenant de différentes voies biosynthétiques, ce qui explique leurs diversités.
Les composés organiques volatils microbiens peuvent être classés selon leurs rôles ou bien selon leurs classes de familles chimiques. En se basant sur ce dernier moyen de classement, nous pouvons distinguer plusieurs familles à savoir, les hydrocarbures, les alcools, les cétones, les aldéhydes, les acides carboxyliques, les esters, les alcaloïdes, les dicétopipérazines, etc.
Les *Bacillus* et les *Burkholderia* sont parmi les meilleurs candidats pour la production des métabolites secondaires, volatils et non volatils, biologiquement actifs. En effet, ces deux genres bactériens sont à l'origine d'une large variété de molécules bioactifs utilisés en agriculture et en application pharmaceutique (Demain and Lancini, 2006). Plusieurs travaux ont été menés pour optimiser la production de métabolites d'intérêts agronomique et pharmaceutique à partir de souches bactériennes et il a été démontré l'importance majeure et l'implication de l'influence des conditions de culture (pH, température, composition du milieu, temps d'incubation…) de la bactérie sur la production des métabolites secondaires.
Dans ce qui suit, nous présentons quelques facteurs affectant la sécrétion des métabolites secondaires ainsi que quelques classes des composés volatils et non volatils microbiens et leurs intérêts biologiques.

1.1. Effets des conditions de culture sur la composition chimique microorganismes

1.1.1. Effet de la composition du milieu de culture

La nature et la concentration des nutriments utilisés dans le milieu de culture ont un effet éminent sur la production des métabolites secondaires. En fait, plusieurs travaux ont montré

que la nature des sources : de carbone, d'azote, ainsi que les additifs tel que le potassium et le phosphore affectent la production des métabolites actifs (Gesheva et al., 2005). De plus, Mellouli et al., 2003 ont constaté que la production d'un dérivé bioactif de dicétopipérazine ; le Cyclo (*L*-Phe, *L*-Pro) à partir d'une nouvelle bactérie du genre *Streptomyces*, est étroitement liée à la nature de la source de carbone utilisé dans le milieu de culture.

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1.1.2. Effet du pH

Outre l'importance de la composition du milieu de culture, plusieurs travaux ont montrés l'implication d'autres facteurs dans la production des métabolites secondaires par les microorganismes. Pour le pH, il a été démontré, depuis longtemps, l'implication et l'influence du pH sur la production de plusieurs métabolites secondaires. En effet, depuis 1973, Iwai et al., ont montré que la production de la cerulenine à partir de *Cephalosporium caeruleus* est énormément affectée par la variation du pH du milieu de culture.

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1.1.3. Effet du temps d'incubation

Chez les bactéries, l'évolution de la production des métabolites secondaires en fonction du temps est un facteur déterminant qui peut être variable d'une espèce à une autre. Par exemple, chez les *Streptomyces*, certaine souche tel *Streptomyces* TN58, la production des biomolécules atteint un maximum après trois jours d'incubation tandis que *Streptomyces*

rochei AK39, la production ne peut atteindre un maximum de production qu'après huit jours (Mellouli et al., 2004 ; Augustine et al., 2005).

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1.2. Les composés organiques microbiens

Les composés organiques microbiens comprennent des molécules de signalisation, des antibiotiques, des pigments, des toxines, des inhibiteurs d'enzymes et des agents anti-tumoraux. Parmi ces composés, on va signaler particulièrement :

- **Les β-Lactames :**

Les β-lactames ou β-lactamines sont des antibiotiques caractérisés par la présence d'un cycle à quatre chainons comprenant une fonction amide dont il est le responsable de l'efficacité de ces molécules. Cette classe d'antibiotique comprend les dérivés de la pénicilline, céphalosporines, céphamycines… qui sont produits par une large variété de champignons et bactéries (*Penicillium* spp., *Aspergillus* spp., *Acremonium* spp., *Streptomyces* cattleya …) (Pelàez, 2006).

Pénicilline G
(*Penicillium notatum*)

Thienamycin
(*Streptomyces cattleya*)

Figure 1. Structures de quelques β-lactames

- **Les macrolides :**

Les macrolides (macrolides antibactériens) sont des molécules à caractère antibiotiques caractérisés par leurs noyaux de lactones de 12 à 16 atomes, souvent munis de deux ou

plusieurs sucres neutres ou aminés. Ils constituent une famille d'antibiotique susceptible de diffuser dans les tissus et au sein des cellules. L'Erytromycine, le composé le plus connu dans cette famille, est un produit de *Saccharopolyspora erythraea,* qui inhibe la biosynthèse de protéines et par suite empêche la croissance de la bactérie (Chen et al., 2008).

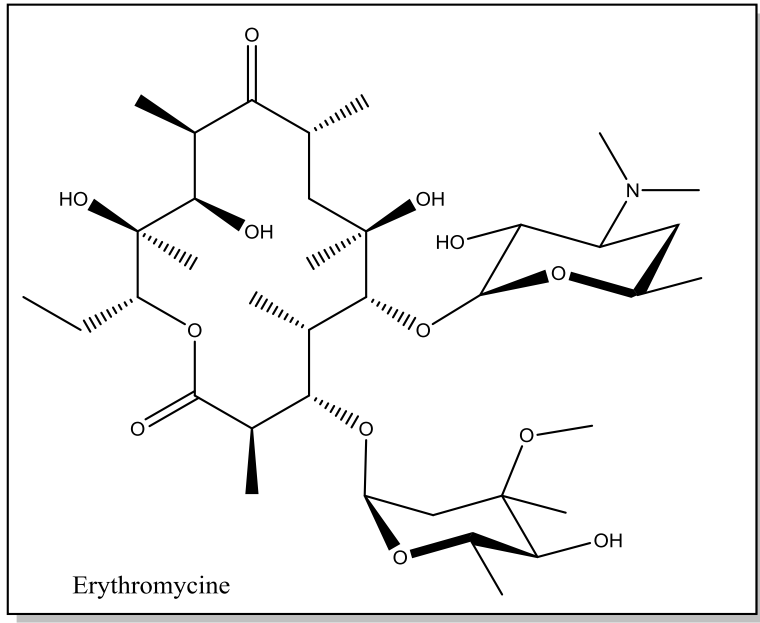


Figure 2. Structure d'Erythromycine (macrolide)

- **Les quinolones :**

Les quinolones présentent une classe des antibiotiques dite à large spectre et qui reçoivent des intérêts croissants vue leurs actions sur de nombreuses bactéries. Ils sont caractérisés par une structure bicyclique, avec un azote en position 1 et un carbonyle en position 4. Cette famille d'antibiotiques est susceptible de diffuser dans l'ensemble de l'organisme, notamment dans la prostate et l'os (Drlica et al., 2009). Parmi ces composés, on cite la burkholone, produit de *Burkholderia* sp. QN15488, qui est un agent cytotoxique puissant (Mori et al., 2007).

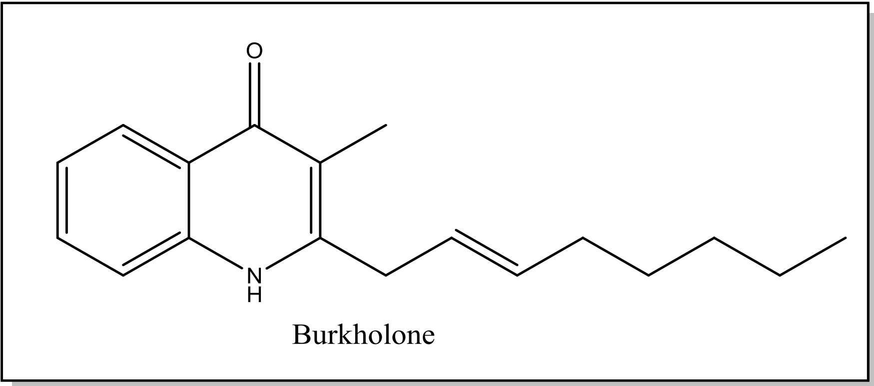


Figure 3. Structure de burkholone (quinolone)

- **Les tétracyclines :**

Ce sont des antibiotiques composés de quatre cycles à six chainons disposés linéairement. Ils sont produits, d'une façon caractéristique, à partir des bactéries du genre *Streptomyces* qui agissent contre des infections bactériennes à Gram positif, négatif, anaérobie et certains autres microbes. L'usage clinique de l'oxy-tétracycline, le chlorotétracycline et d'autres dérivés de la tétracycline est largement répandu aujourd'hui (Strohl 1997).

Tetracycline Chlorotetracycline Oxytetracycline

Figure 4. Structures de quelques tétracylines

- **Les polyènes**

Les polyènes sont des macrolides antifongiques qui se différent des macrolides antibactériens par leurs tailles de noyaux de lactones allant jusqu'à 38 atomes. Ils sont caractérisés aussi par la présence d'une série conjuguée des doubles liaisons. Amphotéricine B, l'unique produit connu par son usage clinique, est émis par *Streptomyces nodosus* et il joue le rôle d'un antifongique caractérisé par sa perméabilité intéressante dans les cellules.

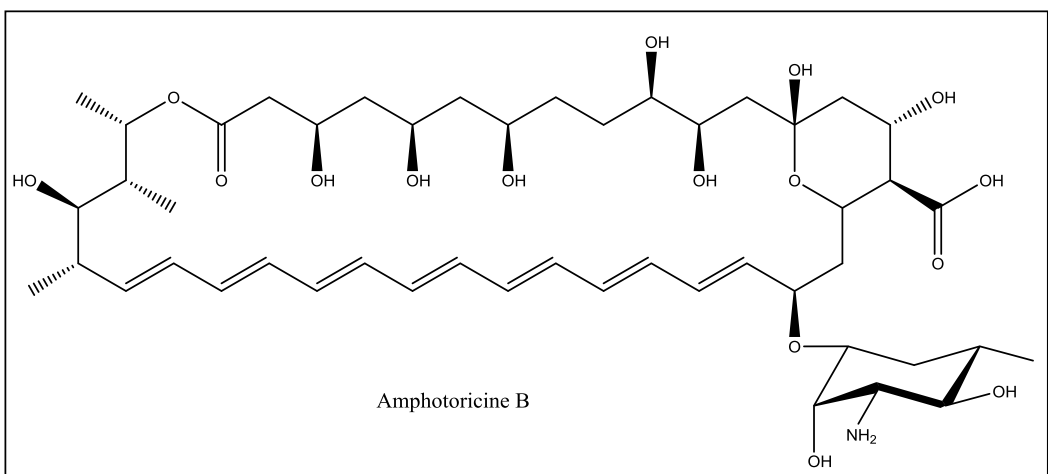


Figure 5. Structures de Amphotéricine B

- **Les dicétopippérazines**

Les dicétopipérazines, les plus petits peptides cycliques, sont connus par leurs propriétés antibiotiques et constituent une nouvelle classe de métabolites secondaires qui sont produits principalement par les champignons, les levures, les bactéries comme les *Streptomyces* et les *Pseudomonas* (Holden et al., 1999, Ben Ameur et al., 2004 ; 2006) et des microorganismes marins (Ri-Ming Huang et al., 2014). Ces cyclopeptides possèdent des activités antimicrobiennes, antitumorales, anti-mutagénique, antivirales et antiprotozoaires (Rhee et al., 2001; Rhee, 2002 ; Rhee, 2004 ; Byun et al., 2003). En plus, les dicétopiperazines présents dans la fraction volatile jouent un rôle très important dans la communication des microorganismes et dernièrement cette classe de peptides cycliques a été classée comme étant des molécules de signalisation intervenant dans les interactions bactérie-bactérie, bactérie-champignons et même bactérie-plante (Barnard et Salmond, 2004; Holden et al., 1999).

Tryprostatine   TAN-1496 C

Figure 6. Structures de quelques dicétopipérazines (CONVM)

3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione   3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione

Figure 7. Structures de quelques dicétopipérazines (COVM)

- **Les hydrocarbures**

Les hydrocarbures sont connus en tant que produits pétroliers et/ou produits des huiles essentielles, mais aussi ils sont produits par les microorganismes via une oxydation destructive des acides gras (Wilkins et Larsen 1995). L'émission de cette famille de composé

dépend essentiellement de la nature du microorganisme utilisé, le stage de maturation du microbe et de l'environnement où il croit. De nombreux hydrocarbures sont connus par leurs interactions avec les plantes et les bactéries et ils sont capables d'induire les réponses des plantes et des bactéries (Kanchiswamy et al., 2015).

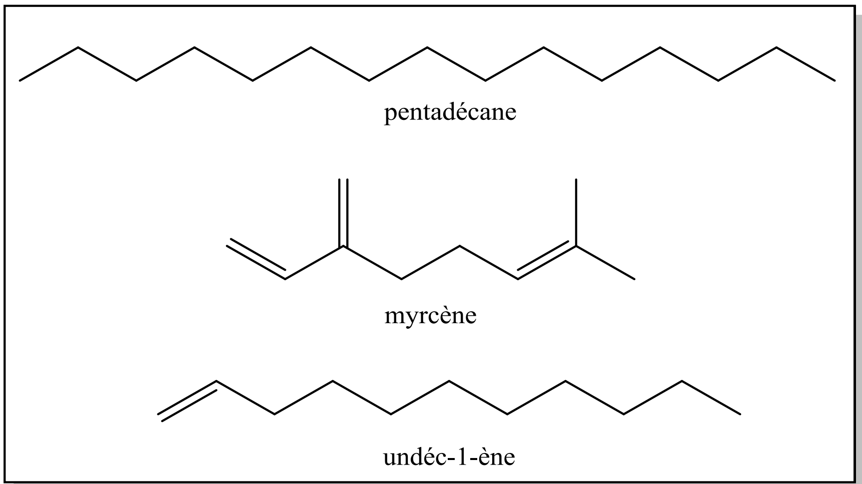


Figure 8. Structures de quelques hydrocarbures

- **Les alcools**

Pendant de nombreuses années, la seule source d'obtention de l'éthanol était à partir de la fermentation des sucres à l'aide des levures. Plusieurs autres alcools sont toujours préparés de la même façon. Ce processus biologique implique la conversation des sucres en acide pyruvique qui lui aussi à son tour peut être converti en de nombreux alcools tels que l'éthanol, butandiol, butanol, etc.

Ces composés sont connus par leurs sécrétions à partir des différents microorganismes et ils sont dotés d'un nombre considérable d'activités biologiques tels que leurs potentiels allelopathiques, activité antifongique, etc. (Kanchiswamy et al., 2015 ; Kishimoto et al., 2007 ; Han et al., 2006).

OH
OH
butan-2,3-diol
HO
hexan-1-ol
OH
octan-3-ol

Figure 9. Structures de quelques alcools

- **Les amines**

Ce sont des composés organiques contenant de l'azote qui sont formées et émises sous formes de métabolites secondaires des plantes, animaux et même des microorganismes (Kataoka 1996 ; Rivers et al., 1992). De nombreuses amines ont une forte odeur désagréable, comme ils sont des puissants irritants pour la peau, les yeux et les voies respiratoires (Lestremau et al., 2001 ; Greim et al., 1998). Ce groupe des composés volatils est issu non seulement suite à la décarboxylation des acides aminés mais aussi suite à une amination des composés carbonylés (Wright et al., 1976). A côté de leurs potentiels toxicologiques, ces composés sont connus par leurs modulations de la croissance bactérienne et la promotion de la croissance des plantes (Velazquez-Becerra et al., 2011).

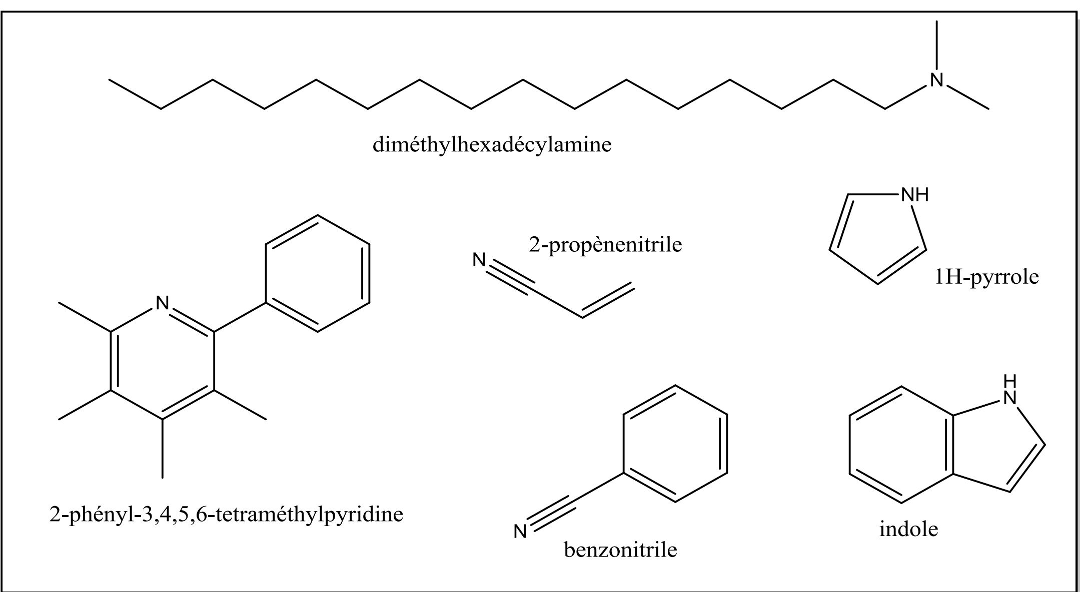


Figure 10. Structures de quelques amines

- **Les composés furaniques**

Les furanes sont des composés hétérocycliques constitués d'un cycle à cinq chainons dont un atome d'oxygène. De nombreux composés furaniques ont été formés et émis sous forme de métabolites secondaires des végétaux et même des microorganismes (Zou et al., 2010 ; Farag et al., 2006). A côté de leurs potentiels toxicologiques, ces composés sont connus par leurs inductions des réponses bactériennes ainsi que par leurs modulations de la croissance bactérienne (Kanchiswamy et al., 2015 ; Zou et al., 2010).

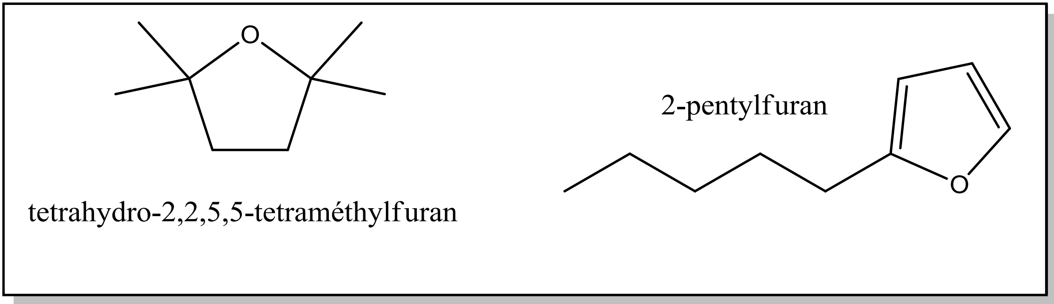


Figure 11. Structures de quelques composés furaniques

- **Les cétones**

Les cétones sont parmi les groupes des composés volatils les plus communs émis par les microorganismes. La production de ces composés dépond essentiellement des espèces étudiées et des milieux de culture utilisés. Ce groupe des composés est issu de la dégradation des acides gras et/ou d'un un processus biochimique tel que la glycolyse (Wilkins et *al.*, 1996 ; Bjurman et *al.*, 1999 ; Wilkins et *al.*, 2000). Ces derniers sont dotés d'un nombre considérable d'activités biologiques, essentiellement l'application dans le domaine agronomique (Scala et *al.*, 2013 ; Schulz and Dickschat 2007; Weise et *al.*, 2014 ; Ryu et *al.*, 2003; Rudrappa et *al.*, 2010).

3-hydroxybutan-2-one (acetoin)
acétophénone
4-méthylheptan-2-one
undécan-2_one
butan-2-one

Figure 12. Structures de quelques cétones

- **Les aldéhydes**

Les composés carbonylés tels que les aldéhydes sont présents partout, dans les plantes, les animaux et les microorganismes. Ils sont connus pour leurs effets irritants et leurs impacts sur la santé. Cependant, des aldéhydes possèdent des intérêts thérapeutiques et agronomiques ; par exemple *O*-anisaldéhyde, acétaldéhyde, propanal et benzaldéhyde.

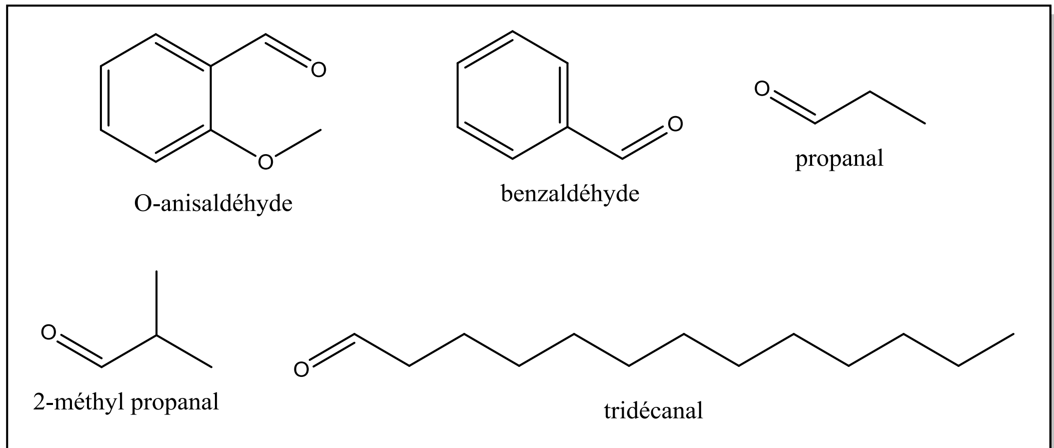


Figure 13. Structures de quelques aldéhydes

- **Les acides carboxyliques**

Les acides carboxyliques sont à la fois des substrats et des produits dans le métabolisme des composés organiques volatils microbiens et plusieurs travaux scientifiques ont montré que divers acides sont formés à partir des acides linoléiques, des triglycérides et des acides aminés (Bjurman et *al.*, 1999). Ces composés et essentiellement les acides carboxyliques à faible masse moléculaire, tel que l'acide isobutyrique, l'acide isovalérique, sont largement utilisés

dans l'application industrielle en tant qu'additifs alimentaires, dans la synthèse organique et dans la production des plastiques (Hekmat et *al.*, 1991 ; Lind et *al.*, 2000 ; Chen and Wu, 2005).

acide 3-hydroxydodécanoique

acide isobutyrique

acide butanoique

acide 2-méthylbutanoique

acide isovalerique

Figure 14. Structures de quelques acides carboxyliques

- **Les esters**

Les microorganismes sont connus par leurs productions des composés oxydés tels que les alcools et les acides. Ces produits peuvent réagir (estérification) entre eux et mènent à la formation des esters. Parmi ces esters, on cite les acétates connus par leurs émissions à partir des espèces de genre *Penicillium* et liés directement au stage de maturation de ce genre (Larsen et Frisvad, 1995). Ces produits sont généralement trouvés dans l'atmosphère des microbes qui se sont révélés interférer négativement avec la production des enzymes liées à la morphogenèse et le développement des mycéliums (Fialho et al., 2011). Ils peuvent inhiber considérablement le développement des bactéries et champignons pathogènes pour l'être humain et les plantes (Strobel et al., 2011).

acetate d'isopentyle
(Isoamyl acetate)

acétate d'éthényle

acétate d'éthyle

octanoate d'éthyle

Figure 15. Structures de quelques esters

2. Activités biologiques des métabolites sécondaires microbiens

Les microorganismes sont des remarquables agents de production des métabolites sécondaires bioactifs et on leur doit la production de nombreuses molécules organiques obtenues par fermentation et utilisées pour leurs propriétés fonctionnelles dans des domaines extrêmement variés. Parmi ces molécules, on peut citer des molécules possédant des propriétés anti-microbienne, anticancéreuse (Holden et al., 1999 ; Kanchiswamy et al., 2015 ; Zou et al., 2010 ; Velazquez-Becerra et al., 2011) ainsi que leurs potentiels de germination, de croissance et de productivité des plantes (Kanchiswamy et al., 2015 ; Rudrappa et al., 2010 ; Scala et al., 2013 ; Schulz and Dickschat 2007; Weise et al., 2014 ; Zou et al., 2010).

Le tableau 1 suivant regroupe les structures de quelques composés bioactifs.

Tableau 1. Exemples des molécules microbiennes bioactives

Molécule	Activité biologique
	Anti-α-glucosidase (Kwon et al., 2000)

Phenylahistine	Anticancéreux (Martins and Carvalho, 2007)
	Anticancéreux (Folkes et al., 2001)
Azonazine	Anti-inflammatoire (Martins and Carvalho, 2007)
	Potentiel de germination (Kanchiswamy et al., 2015)

3. Etude bibliographique de *Bacillus megaterium* AGN01

3.1. Généralités

Le genre *Bacillus* regroupe environ 36 espèces de bactéries à Gram positif de la famille des *Bacillacées* (*Bacillaceae*). Les *Bacillus* sont ubiquitaires car leurs exposition d'une large

gamme de capacités physiologiques ainsi leurs spores leurs confèrent une grande résistance et leurs permet de se trouver dans divers niches. Ils sont largement répandus dans la nature ; On en trouve dans les sols, sur les plantes, l'eau, poissons… (Vary, 1994).

De nombreuses études ont montré que ce genre est très utilisé dans de nombreux procédés médicaux, pharmaceutiques, agricoles et industriels vue leur capacité à produire une large série d'enzymes, des antibiotiques et d'autres métabolites secondaire (Peter et al., 1996). Parmi ces métabolites, on cite les dicetopiperazine, les alcools etc… (Farag et al., 2006 ; Zou et al.,2007).

Les principaux métabolites de quelques espèces ont été recensés dans le tableau 2 suivant :

Tableau 2. Structures de quelques composés isolés d'espèces du genre *Bacillus*

Famille	Composé	Espèce
Peptide	Pumilacidins B : X = Val, R = C_{12}-iso Pumilacidins D : X = Val, R = C_{14}-iso	*Bacillus pumilus* (Melo et al., 2009)
Hydrocarbures	Nonane	*Bacillus simplex* (Gu et al., 2007)
	Undécène	*Bacillus. ssp* (Farag et al., 2006)
	Cyclohexène	*Bacillus simplex* (Gu et al., 2007)

Macrolide	Aurantinine A	*Bacillus aurantinus* (Nishikiori et al., 1978)
	Macrolactine H	*Bacillus* sp. (Nagao et al., 1997)
	Macrolactine L	*Bacillus* sp. (Nagao et al., 1997)
alcools	butan-2,3-diol	*Bacillus amyloliquefaciens* (Rudrappa et al., 2010)
	3-méthylbutanol	*Bacillus amyloliquefaciens* (Farag et al., 2006)
	3,4-Diméthylhex-5-èn-3-ol	*Bacillus pumilus* (Wei-wei et al., 2008)
	Phénol	*Bacillus simplex* (Gu et al., 2007)

Acide Carboxyliques	acide glyoxilique	*Bacillus*. ssp (Farag et al., 2006)
	Acide 2-éthylbutanoique	*Bacillus* ssp (Farag et al., 2006)
	Acide acétique	*Bacillus* ssp (Schulz and Dickschat, 2007)
Esters	phtalate de diéthyle	*Bacillus* ssp., *Bacillus*. *pumilus* (Wei-wei et al., 2008)
	acétate d'éthyle	*Bacillus amyloliquefaciens* (Farag et al., 2006)
Aldéhydes	phénylacétaldéhyde	*Bacillus* spp (Zou et al.,2007)
	décanal	*Bacillus simplex* (Gu et al., 2007)
	hexa-2,4-diènal	*Bacillus amyloliquefaciens* (Farag et al., 2006)

Cétones	acetoin	*Bacillus subtilis* (Ryu et al., 2003)
	2-hydroxypentan-3-one	*Bacillus* amyloliquefaciens (Farag et al., 2006)
	acétophénone	*Bacillus simplex* (Gu et al., 2007)
Amines	Méthylpyrazine	*Bacillus* spp. (Zou et al.,2007)
	butanamine	*Bacillus* spp. (Zou et al.,2007)
Composés soufrés	diméthyldisulfide	*Bacillus cereus* (Huang et al., 2012)
	bezothiazole	*Bacillus* spp. (Zou et al.,2007)
	diméthyltrisulfide	*Bacillus* spp (Farag et al., 2006)
Dicétopipérazines	Bacillusamide A	*Bacillus* sp. (Yonezawa et al., 2011)

	Bacillusamide B	*Bacillus* sp. (Yonezawa et al., 2011)
Composés furaniques	2-pentylfurane	*Bacillus amyloliquefaciens* (Farag et al., 2006)
	Tétrahydro-2,5-diméthylfurane	*Bacillus amyloliquefaciens* (Farag et al., 2006)

L'espèce *Bacillus megaterium* « du Latin signifiant grosse bête – en anglais ; big beast » est l'une des plus grosses bactéries ; d'environ 100 fois plus grande que *E. Coli*. Principalement une bactérie du sol, *B. megaterium* a été également trouvé dans une variété d'habitats, tels que l'eau de mer, les sédiments, les rizières, le poisson (Vary, 1994 ; Vary et al., 2007). *B megaterium* a plusieurs applications médicale, pharmaceutique, agronomique et même industrielle (Vary et al., 2007).

La classification systématique de *B. megaterium* est présentée par le classement suivant :

Règne *Bacteria*

Embranchement *Firmicutes*

Classe *Bacilli*

Ordre *Bacillalis*

Famille *Bacillaceae*

Genre *Bacillus*

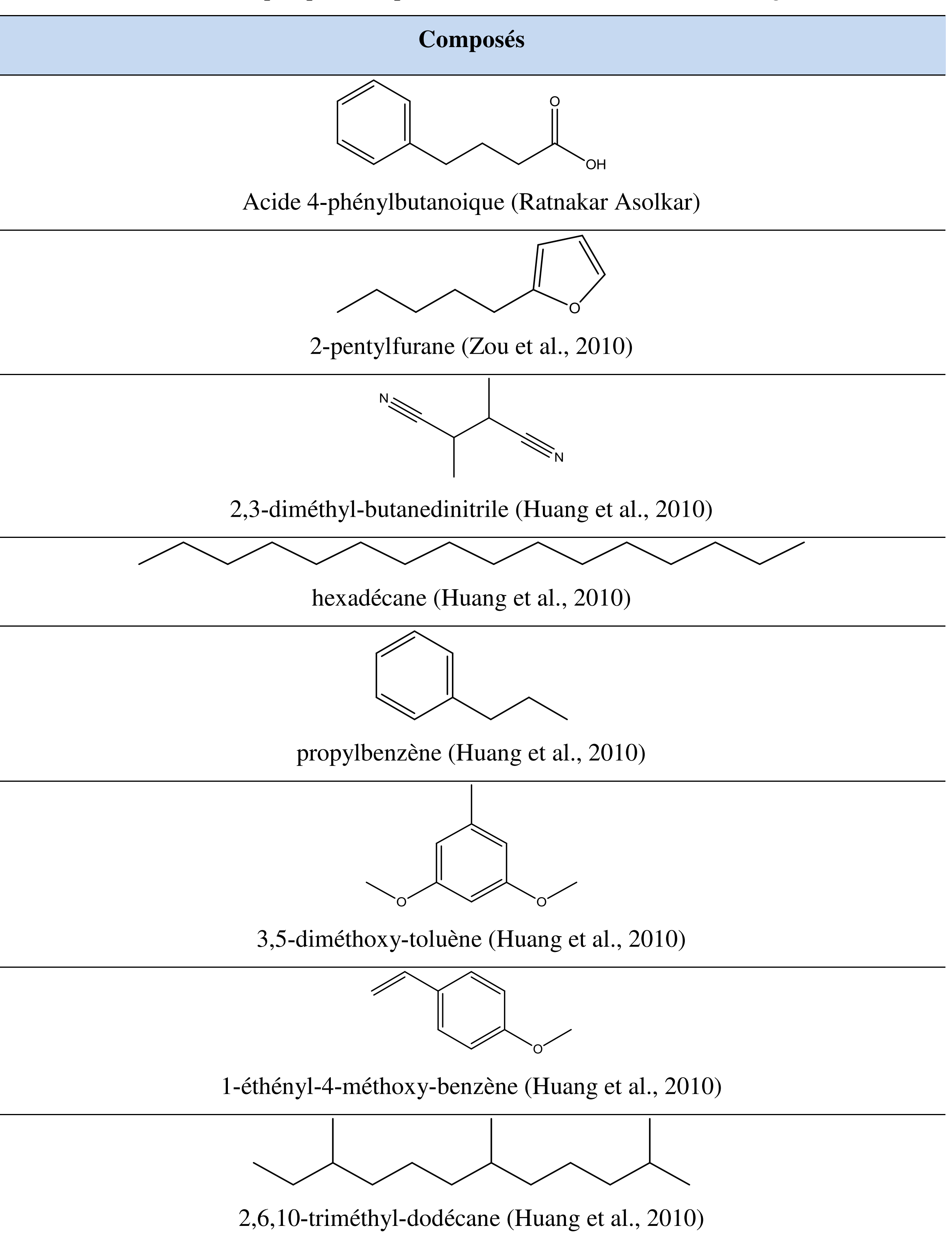
Espèce *Bacillus megaterium* AGN01

3.2. Travaux antérieurs

Les recherches bibliographiques que nous avons effectuées sur la bactérie *B. megaterium* ont montré qu'elle reste très peu étudiée. Ces mêmes recherches ont montré que cette espèce est

douée en composés aromatique volatils. Le tableau 3 suivant regroupe les structures de quelques composés isolés de *B. megaterium.*

Tableau 3. Structures de quelques composés isolés de la bactérie *Bacillus megaterium.*



Composés
Acide 4-phénylbutanoique (Ratnakar Asolkar)
2-pentylfurane (Zou et al., 2010)
2,3-diméthyl-butanedinitrile (Huang et al., 2010)
hexadécane (Huang et al., 2010)
propylbenzène (Huang et al., 2010)
3,5-diméthoxy-toluène (Huang et al., 2010)
1-éthényl-4-méthoxy-benzène (Huang et al., 2010)
2,6,10-triméthyl-dodécane (Huang et al., 2010)

4. Etude bibliographique de *Burkholderia* sp. AGN02

4.1. Généralités

Le genre *Burkholderia*, β-protéobactérie, comprend plus de 60 espèces résidant divers niches écologiques (Paganin et al., 2011). Les espèces bactériennes du genre *Burkholderia* sont des organismes omniprésents dans le sol, la rhizosphère, les animaux, l'eau, les insectes, les hôpitaux et l'être humain infecté (Coenye et al., 2001 ; Coenye and Vandamme, 2003). La diversité naturelle des membres de ce genre indique que leurs interactions avec leurs hôtes sont complexes et souvent peuvent être contradictoires. Traditionnellement, les espèces du genre *Burkholderia* ont été connues comme des agents pathogènes non seulement pour les plantes mais aussi pour les animaux et l'être humain (Paganin et al., 2011 ; Vial et al., 2011). Par exemple, *B. glumae* provoque la pourriture des graines de riz (Jeong et al., 2003), de plus, *B. pseudomallei* et *B. mallei* sont les espèces les plus connus de ce genre qui sont pathogènes pour l'être humain (Cheng and Currie, 2005).

En revanche, les espèces du genre *Burkholderia* sont connus par leurs sécrétions des produits extracellulaires tels que les enzymes, les antibiotiques, les alcools, les phénols, les acides carboxyliques, les quinolones, les dicétopipérazines…

Le Tableau 4 regroupe les structures de quelques composés isolés d'espèces du genre *Burkholderia*.

Tableau 4. Structures de quelques composés isolés d'espèces du genre *Burkholderia*

Famille	Composé	Espèce
Composés soufrés	diméthydisulfide	*Burkholderia tropica* (Tenorio-Salgado et al., 2013)
	S-Méthyl méthanethiosulphonate	*Burkholderia ambifaria* (Groenhagen et al., 2013)
Hydrocarbures	méthylcyclohexane	*Burkholderia tropica* (Tenorio-Salgado et al., 2013)

	Toluène	*Burkholderia tropica* (Tenorio-Salgado et al., 2013)
	p-Xylène	*Burkholderia tropica* (Tenorio-Salgado et al., 2013)
Cétones	hexan-3-one	*Burkholderia ambifaria* (Groenhagen et al., 2013)
	1-phénylpropan-1,2-dione	*Burkholderia ambifaria* (Groenhagen et al., 2013)
	Acétophénone	*Burkholderia ambifaria* (Groenhagen et al., 2013)
Alcools et Phénols	heptan-4-ol	*Burkholderia ambifaria* (Groenhagen et al., 2013)
	1-phénylpropanol	*Burkholderia ambifaria* (Groenhagen et al., 2013)

Esters et Lactones	3-Méthyl-4-butanolide	*Burkholderia ambifaria* (Groenhagen et al., 2013)
	Acétate d'isobornyle	*Burkholderia ambifaria* (Groenhagen et al., 2013)
Chromanes	2-hydroxychroman-4-one	*Burkholderia* sp. MSSP (Kang et al., 2004)
Oxazoles	Templazole A	*Burkholderia* sp. A 396 (Asolkar et al., 2011)
	Templazole B	*Burkholderia* sp. A 396 (Asolkar et al., 2011)
Pyrazines	Tétraméthylpyrazine	*Burkholderia ambifaria* (Groenhagen et al., 2013)
		Burkholderia ambifaria (Groenhagen et al.,

	2-Méthyl-5-isopropylpyrazine	2013)
Dicétopipérazines		*Burkholderia cepacia* CF-66 (Wang et al., 2010)
		Burkholderia cepacia CF-66 (Wang et al., 2010)
Quinolines et Quinolones	2-Heptyl-3-méthylquinolin-4-ol	*Burkholderia ambifaria* (Vial et al., 2008)
	2-Hepthylquinolin-4-ol	*Burkholderia ambifaria* (Vial et al., 2008)
	Burkholone	*Burkholderia* sp. QN15488 (Mori et al., 2007)

L'espèce *Burkholderia* sp. est une nouvelle Gram négative, bactérie extraite pour la première fois à partir du sol Français par nos collègues de l'industrie AGRONUTRITION.

La classification systématique de *Burkholderia* sp. est présentée par le classement suivant :

Règne *Bacteria*

Embranchement *Proteobacteria*

Classe *Beta Proteobacteria*

Ordre *Burkholderiales*

Famille *Burkholderiaceae*

Genre *Burkholderia*

Espèce *Burkholderia* sp. AGN02

Références

A

Asolkar, R., Koivunen, M., Marrone, P., Lucia, A., Kreylos, C., Huang, H. 2011. Isolated bacterial strain of the genus *Burkholderia* and pesticidal Metabolites There from. *U.S. Patent. US 20110207604A1*

Augustine, S., K., Bhavsar, S., P., Kapadnis, B., P. 2005. Production of a growth dependent metabolite active against dermatophytes by *Streptomyces rochei* AK 39. *Indien J. Med. Res*. 121:164-170.

B

Barnard, A., M. L., Salmond, G., P., C. 2004. Quorum sensing: the complexities of chemical communication between bacteria. *Complexus* 5, 87-101

Ben Ameur-Mehdi, R., Mellouli, L., Chabchoub, F., Fotso, S., Bejar, S. 2004. Purification and Structure Elucidation of two Biologically Active Molecules from a new Isolated *Streptomyces* sp. US24 strain. *Chem of Nat Comp*. 40, 510-513.

Ben Ameur Mehdi R., Sioud S., Fourati Ben Fguira L., Bejar S. and Mellouli, L. 2006. Purification and structure determination of four bioactive molecules from a newly isolated *Streptomyces* sp. TN97 strain. *Process Biochem.* 41, 1506-1513.

Bjurman, J. 1999. Release of MVOCs from microorganisms. In Organic Indoor Air Pollutants - Occurrence, Measurement, Evaluation. *Ed. T. Salthammer* 259-273

Byun, H., G., Zhang, H., Mochizuki, M., Adachi, K., Shizuri, Y., Lee, W., J., Kim, S., K., J. 2003. *Antibiot*. 56, 102.

C

Chen, G., Q., Wu, Q. 2005. Microbial production and applications of chiral hydroxyalkanoates. *Appl Microbiol Biotechnol* 67, 592-599.

Chen Y, Deng W, Wu JQ, Qian JC, Chu J. 2008. Genetic modulation of the overexpression of tailoring genes *eryK* and *eryG* leading to the improvement of erythromycin a purity and production in *Saccharopolyspora erythraea* fermentation. *Appl Environ Microbiol* 74, 1820–1828

Cheng, A., C., Currie, B., J. 2005. Melioidosis: Epidemiology, pathophysiology, and management. *Clin. Microbiol. Rev*. 18, 383-416.

Coenye, T., Vandamme, P., Govan, J., R., LiPuma, J., J. 2001. Taxonomy and identification of the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* 39, 3427-3436.

Coenye, T., Vandamme, P. 2003. Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Env Microbiol*. 5, 719-729.

D

Demain, A., L., and Lancini, G. 2006. Bacterial Pharmaceutical Products. The Prokaryotes. A handbook on the biology of bacteria. Dowrkin, M., Falkow, S., Rosenberd, E., Schleifer, K., Stackebrandt, E. *3rd ed. Springer, New York, USA*. 1 , 812-833.

Drlica, K., Hiasa, H., Kerns, R., Malik, M., Mustaev, A., Zhao, X. 2009. Quinolones: Action and Resistance Updated . *Curr. Top. in Med. Chem.* 9, 981-998.

F

Farag, M., A., Ryu, C,. M., Summer, L., W., Pare, P., W. 2006. GC-MS SPME profiling of rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants. *Phytochem.* 67, 2262–2268.

Fialho, M., B., Romanholo Ferreira, L., F., Rosim Monteiro, R., T., Pascholati, S., F. 2011. Antimicrobial volatile organic compounds affect morphogenesis-related enzymes in *Guignardia citricarpa*, causal agent of citrus black spot. *Biocontr.Sci.Technol*. 21, 797-807.

Folkes, A.; Roe, M. B.; Sohal, S.; Golec, J.; Faint, R.; Brooks, T.; Charlton, P. 2001. *Bioorg. Med. Chem. Lett*. 11, 2589.

G

Gesheva V., Ivanova V., Gesheva R. 2005. Effects of nutrients on the production of AK-111-81 macrolide antibiotic by *Streptomyces hygroscopicus*. *Microbiol. Res*. 160, 243-248.

Groenhagen, U., Baumgartner, R., Bailly, A., Gardiner, A., Eberl, L., Schulz, S., Weisskopf, L. 2013. Production of Bioactive Volatiles by Different *Burkholderia ambifaria* strains. *J.Chem.Ecol*. 39, 892-906.

Greim, H., Buty, D., Klimisch, H., J.,Oeben-Negele, M., Ziegler-Skylakakis, K. 1998. Toxicity of aliphatic amines: structure-activity relationship. *Chemosphere* 36, 271-195.

Gu, Y., Q., Mo, M., H., Zhou, J., P., Zou, C., S., Zhang, K., Q. 2007. Evaluation and identification of potential organic nematicidal volatiles from soil bacteria. *Soil Biol. Biochem.* 39, 2567-2575.

H

Han, S., H., Lee, S., J., Moon, J., H., Park, K., H., Yang, K., Y., Cho, B., H., Kim, K., Y., Kim, Y., W., Lee, M., C., Anderson, A., J., Kim, Y., C. 2006. GacS-dependent production of 2*R*, 3*R*-butanediol by *Pseudomonas chlororaphis* O6 is a major determinant for eliciting systemic resistance against *Erwinia carotovora* but not against *Pseudomonas syringae* pv. tabaci in tobacco. *Mol.Plant-Microbe Interact*. 19, 924-930.

Hekmat, M., Smith, R., G. 1991. Determination of low molecular weight organic acids collected on silica gel air sampling tubes by using ion-exclusion chromatography. *Am Ind Hyg Assoc J*. 52, 332-335

Holden, M., T., G., Chhabra, S., R., Denys, R., Stead, P., Bainton, N., J., Hill, J., P., Manefield, M., Kumar, N. 1999 Quorum sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other Gram-negative bacteria. *Mol Microbiol* 33,1254-1266.

Huang, Y., Xu, C., K., Ma, L., Zhang, K., Q., Duan, C., Q., Mo, M., H. 2010. Characterisation of volatiles produced from *Bacillus megaterium* YFM3.25 and their nematicidal activity against Meloidogyne incognita. *Eur J Plant Pathol*. 126, 417-422

Huang, C., J., Tsay, J., F., Chang, S., Y., Yang, H., P., Wu, W., S., Chen, C., Y. 2012. Dimethyldisulfide is an induced systemic resistance -elicitor produced by Bacillus cereusC1L. *Soc. Chem. Ind.*

I

Iwai, Y., Awaya, J., Kesado, T., Yamada, H., Omura, S., Hat, T. 1973. Selective fermentation of cerulenin by *Cephalosporin caeruleus*. *J. Ferment. Technol.* 51, 575-578.

J

Jeong, Y., Kim, J., Kim, S., Kang, Y., Nagamatsu, T., Hwang, I. 2003. Toxoflavin produced by *Burkholderia glumae* causing rice grain rot is responsible for inducing bacterial wilt in many field crops. *Plant Dis*. 87, 890-895.

K

Kanchiswamy, C., N., Malnoy, M., Maffei, M., E. 2015. Chemical diversity of microbial volatiles and their potential for plant growth and productivity. *Front. Plant Sci*. 6,151

Kang, J., G., Shin, S., Y., Kim, M.J., Bajpai, V., Maheshwari, D., K., Kang, S., C. 2004. Isolation and anti-fungal activities of 2-hydroxymethyl-chroman-4-one produced by *Burkholderia* sp. MSSP. *J Antibiot* 57, 726–731.

Kataoka, H. 1996. Derivatization reactions for the determination of amines by gas chromatography and their applications in environmental analysis. *J Chromatogr*. 733, 19-34

Kishimoto, K., Matsui, K., Ozawa, R., Takabayashi, J. 2007. Volatile 1-octen-3-ol induces a defensive response in *Arabidopsis thaliana*. *J.Gen.Plant Pathol.* 73, 35-37.

Kwon, O. S.; Park, S. H.; Yun, B. S.; Pyun, Y. R.; Kim, C. J. 2000, Cyclo(dehydroala-L-Leu), an alpha-glucosidase inhibitor from *Penicillium* sp. F70614. *J. Antibiot*. 53, 954.

L

Larsen, T., Frisvad, J. 1995. Chemosystematics of *Penicillium* based on profiles of volatile metabolites. *Mycological Research* 99, 1167-1174.

Lestremau, F., Desauziers, V., Fanlo, J., L. 2001. Formation of artefacts during air analysis of volatile amines by solid phase micro extraction. *The Analyst* 126,1969-1973.

Lind, H., Jonsson, H., Schnürer, J. 2005. Antifungal effect of dairy propionibacteria-contribution of organic acids. *Int. J. Food Microbiol.* 98, 157-165

M

Martins MB, Carvalho I. 2007. Diketopiperazines: Biological activity and synthesis. *Tetrahedron*. 63, 9923-9932.

Mellouli L., Ben Ameur-Mehdi, R., Sioud, S., Salem M. and Bejar S. 2003. Isolation, purification and partial characterization of antibacterial activities produced by a new isolated *Streptomyces sp.* US24 strain. *Res. in Microbiol.* 154, 345-352.

Mellouli L., Karray-Rebai .I, Sioud S., Ben Ameur-Mehdi R., Naili, B., Bejar, S. 2004. Efficient transformation procedure of a newly isolated *Streptomyces* sp. TN58 strain producing antibacterial activities. *Curr. Microbiol.* 49, 400-406.

Melo, F., M., P., Fiori, M., F., Moraes, L., A., B., Silva-Stenico, M., S., Scramin, S., Teixeira, M., A., Melo, I., S. 2009. *Sci. Agric.* 66, 583.

Mori, T., Yamashita, T., Furihata, K., Hayakawa, Y., Shin-ya, K. 2007. Burkholone, a new cytotoxic antibiotic against IGF-I dependent cells from *Burkholderia* sp.. *J. Antibiot.* 60, 713-716.

N

Nishikiori, T., Masuma, R., Oiwa, R., Katagiri, M., Awaya, J., Iwai, Y., Omura, S. 1978. Aurantinin, a new antibiotic of bacterial origin. *J. Antibiot.* 31, 525-532.

P

Paganin, P., Tabacchioni, S., Chiarini, L. 2011. Pathogenicity and biotechnological applications of the genus *Burkholderia*. *Cent. Eur. J. Biol.* 6, 997-1005.

Peláez F. 2006. The historical delivery of antibiotics from microbial natural products-can history repeat?. *Biochem Pharmacol.* 71, 981-90.

Peter, C., Turnbull, B. 1996. *Bacillus*. Baron, S., editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston.

R

Rhee, K., H., Choi, K., H., Kim, C., J., Kim, C., H. 2001. Identification of *Streptomyces* sp. AMLK-335 producing antibiotic substance inhibitory to VRE (vancomycin-resistant enterococci). *J. Micribiol. Biotechnol* 11, 469–74

Rhee, K., H. 2002. Isolation and characterization of *Streptomyces* sp. KH-614 producing anti-VRE (vancomycin-resistant enterococci) antibiotics. *J. Gen. Appl. Microbiol* 48,321–7.

Rhee, K., H. 2004. Cyclic dipeptides exhibit synergistic, broad spectrum antimicrobial effects and have anti-mutagenic properties. *Int. J. Antimicrob. Agents*. 24, 423-427.

Rivers, J., C., Pleil, J., D., Wiener, J., W. 1992. Detection and characterization of volatile organic compounds produced by indoor air bacteria. *J Expo Anal Env Epid suppl.*1, 177-188

Rudrappa, T., Biedrzycki, M., L., Kunjeti, S., G., Donofrio, N., M., Czymmek, K., J., Pare, P., W., Bais, H., P. 2010. The rhizobacterial elicitor acetoin induces systemic resistance in *Arabidopsis thaliana. Commun. Integr. Biol.* 3, 130-138.

Ryu, C., M., Farag, M., A., Hu, C., H., Reddy, M., S., Wei, H., X., Pare, P., W., Kloepper, J., W. 2003. Bacterial volatiles promote growth in Arabidopsis. *Proc. Natl. Acad. Sci. USA*. 100, 4927-4932.

S

Scala, A., Allmann, S., Mirabella, R., Haring, M.A., and Schuurink, R., C. 2013. Green Leaf Volatiles: A Plant's Multifunctional Weapon against Herbivores and Pathogens. *Int.J.Mol.Sci.* 14, 17781-17811.

Schulz, S., and Dickschat, J., S. 2007. Bacterial volatiles: the smell of small organisms. *Nat.Prod.Rep*. 24, 814-842.

Strobel, G., Singh, S., K., Riyaz-Ul-Hassan, S., Mitchell, A., M., Geary, B., Sears, J. 2011. An endophytic/pathogenic *Phoma* sp from creosote bush producing biologically active volatile compounds having fuel potential. *Fems Microbiol.Lett*. 320, 87-94.

Strohl, W., R. 1997. Biotechnology of antibiotics, *2nd edn. Marcel Dekker, New York.*

T

Tenorio-Salgado S., Tinoco, R., Vazquez-Duhalt, R., Caballero-Mellado, J., Perez-Rueda, E. 2013. Identification of volatile compounds produced by the bacterium Burkholderia tropica that inhibit the growth of fungal pathogens, *Bioengineered*, 4, 236-243,

V

Vary, P., S. 1994. Prime time for *Bacillus megaterium. Microbiology* 140, 1001-1013.

Vary, P., S., Biedendieck, R., Fuerch, T., Meinhardt, F., Rohde, M., Deckwer, W., D., Jahn, D. 2007. *Bacillus megaterium* - from simple soil bacterium to industrial protein production host. *Appl Microbiol Biotechnol*. 76, 957-967.

Vial, L., Lépine, F., Milot, S., Groleau, M. C., Dekimpe, V., Woods, D. E., Déziel, E. 2008. Burkholderia pseudomallei, B. thailandensis, and B. ambifaria Produce 4-Hydroxy-2-

Alkylquinoline Analogues with a Methyl Group at the 3 Position That Is Required for Quorum-Sensing Regulation. *J. Bacteriol.* 5339-5352.

Vial, L., Chapalain, A., Groleau, M. C., Déziel, E. 2011. The various lifestyles of the Burkholderia cepacia complex species: a tribute to adaptation. *Environ. Microbiol.* 13, 1-12.

Velazquez-Becerra, C., Macias-Rodriguez, L., I., Lopez-Bucio, J., tamirano-Hernandez, J., Flores-Cortez, I., Valencia-Cantero, E. 2011. A volatile organic compound analysis from *Arthrobacter agilis* identifies dimethylhexadecylamine, an amino-containing lipid modulating bacterial growth and *Medicago sativa* morphogenesis in vitro. *Plant and Soil.* 339, 329-340.

W

Wang, J., H., Quan, C., S., Qi, X., H., Li, X., Fan. S., D. 2010. Determination of diketopiperazines of *Burkholderia cepacia* CF-66by gas chromatography–mass spectrometry. *Anal. Bioanal. Chem.*396,1773-1779

Weise, T., Thuermer, A., Brady, S., Kai, M., Daniel, R., Gottschalk, G., Piechulla, B. 2014. VOC emission of various *Serratia* species and isolates and genome analysis of *Serratia plymuthica* 4Rx13. *Fems Microbiol.Lett.* 352, 45-53

Wilkins, K., Larsen, K. 1995. Variation of volatile organic compound patterns of mold species from damp buildings. *Chemosphere.* 31, 3225-3236.

Wilkins, K. 1996. Volatile metabolites from actinomycetes. *Chemosphere* 32, 1427-1434.

Wilkins, K., Larsen, K., Simkus, M. 2000. Volatile metabolites from mold growth on building materials and synthetic media. *Chemosphere* 41,437-446

Wright, J., L., C., Vining, L., C., 1976. Secondary metabolites derived from non-aromatic amino acids. *Filamentous Fungi* 2, 475-502.

Y

Yonezawa, K., Yamada, K., Kouno, I. 2011. New diketopiperazine derivatives isolated from sea urchin-derived *Bacillus* sp. *Chem. Pharm. Bull.* 59, 106-108.

Z

Zou, C., S., Mo, M., H., Gu, Y., Q., Zhou, J., P., Zhang, K., Q. 2007. Possible contribution of volatile-producing bacteria in soil fungi-stasis. *Soil Biol. Biochem.* 39, 2371–-2379.

Zou, C., Li, Z., Yu, D. 2010. *Bacillus megaterium* Strain XTBG34 Promotes Plant Growth by Producing 2-Pentylfuran. *J. Microbiol.* 48, 460-466.

Chapitre II : Etude de l'effet du nutriment sur la composition chimique de *Burkholderia* sp. et sur les activités biologiques

Introduction:

Les microorganismes sont utilisés depuis l'antiquité, par les êtres humains, pour la fabrication des produits comme le pain, le fromage, la bière, etc. Plus récemment, et avec l'essor de la biotechnologie, les êtres humains ont commencé à se servir de ces microorganismes en tant que des agents remarquables de production de nombreuses molécules bioactives et utilisées pour leurs propriétés fonctionnelles dans des domaines extrêmement variés allant de l'application agronomique à l'application pharmaceutique. Parmi ces molécules, on peut citer les hydrocarbures, les alcools, les aldéhydes, les acides organiques, les acides aminés, les dicétopipérazines, etc. Il a été démontré l'énorme influence des conditions de culture sur la production bactérienne des métabolites secondaires. En effet, la recherche de nouvelles molécules actives à partir des microorganismes est étroitement liée aux genres des souches étudiées ainsi qu'aux conditions de culture des bactéries.

Les travaux de recherche du présent chapitre s'inscrivent dans le cadre de la recherche de nouvelles molécules bioactives à partir de microorganismes. Nous nous sommes intéressés à l'étude de l'effet de nature de source de carbone (nutriment) sur la composition chimique de *Burkholderia* sp. et sur les activités biologiques de différents extraits obtenus de la même bactérie. Ainsi, pour plus de clarté dans la présentation, il est important de noter que les activités ciblées sont : l'activité anti-inflammatoire (anti-5-lipoxygénase), activité anti-xanthine oxydase, activité anti-acétylcholinestérase, l'activité anti-diabétique (anti-α-amylae), la cytotoxicité (MCF-7, HCT-116, OVCAR et IGROV) ainsi que le potentiel allélophatique des différents extraits obtenus. Les résultats obtenus ainsi que leurs discussions seront présentées en deux parties comme suit :

- La première portera sur l'étude de l'effet des sources de carbone sur la production des substances volatiles microbiennes à partir de *Burkholderia* sp. Au cours de cette partie, nous nous sommes intéressées à l'étude de l'effet de la nature des sources de carbone (dextrose et glycérol) sur la production des métabolites secondaires volatils à partir de *Burkholderia* sp. Au cours de cette partie, nous avons utilisés l'extraction liquide-liquide avec des solvants à polarité croissante permettant l'obtention de quatre extraits. Ces extraits ont été ensuite analysés directement et après silylation par la chromatographique gazeuse couplée à la spectrométrie de masse à haute résolution. Cette partie de thèse fait l'objet d'une publication qui est actuellemnt soumise dans le journal « *International Journal of Medical Microbiology* ».
- La deuxième partie portera sur l'étude de l'effet de la nature des sources de carbone utilisée dans la culture de *Burkholderia* sp. sur les activités biologiques des différents extraits

obtenus. Cette partie de thèse fait l'objet d'une publication qui est actuellemnt soumise «*World Journal of Microbiology and Biotechnology*».

Partie A: GC-HRMS based metabolomics analysis of chemical productions from a new *Burkholderia* sp. AGN02

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Summary

Bacteria communicate within and with other organisms such as fungi and plant by emission of a diffusible volatile low-molecular-weight chemicals that serve as chemical windows through which the information bacterial activities is released. In this study, the volatiles produced by a *Burkholdeia* sp. strain, isolated from an agricultural soil samples, were analyzed, and the effect of the use of different carbon sources (dextrose and glycerol), as nutriment, on the production of volatile organic (Vocs) were also assessed. Gas chromatography-high resolution mass spectrometry (GC-HRMS) analysis of extracts (cyclohexane, dichloromethane, ethyl acetate and n-butanol) with and without derivatization reaction showed that the blend volatiles produced were influenced by the use of different carbon sources. According to the obtained results, more than 60 compounds, belonging to a large variety of compound classes such as hydrocarbons, alcohols, ketones, esters, carboxylic acids, and diketopiperazines, were identified including 3-hydroxy-2-butanone, 3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione, 1-(3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)pyrimidine-2,4(1*H*,3*H*)-dione and *N,N*-dimethyldodecan-1-amine. All detected compounds have never previously been reported as a secondary metabolite of *Burkholdeia* sp.

Keywords

Burkholderia sp., nutriment, Vocs, GC-HRMS, diketopiperazines.

Introduction

The *Burkholderia* genus consists of over 60 different species that inhabit a variety of niches. The genus contains a number of human, animals and plants pathogens species, such as *B. pseudomallei* the causative agent of the melioidosis but *B. mallei* and *B. cepacia* which has been known as onions pathogens (Sacha et al., 2014). In contrast, certain species of *Burkholderia* are known not only for their potential for plant growth but also for their antagonistic effects towards plant pathogens and recently certain species of *Burkholderia* have proved to be very efficient in biological control (Blom et al., 2011; Kanchiswamy et al., 2015) Indeed, several *Burkholderia* species secrete a large spectrum of biochemical products such as enzymes, siderphores, and volatiles and non-volatile metabolites including fungal plant pathogens, antibiotics (Silvia et al., 2013), enacyloxins (Mahenthiralingam et al., 2011), quinoline (Vial et al., 2008), pyrrolonitrin (El-Banna and Winkelmann 1998), toxins such as rhizobitoxine (Mitchell et al., 1986) and bongkrekic acid (Moebius et al., 2012).

Among the volatile compounds produced by *Burkholderia* species, the most important and well documented is the diketopiperazine derivatives, signal molecules which play an important role in signal transduction of *Burkholderia* species. In addition to diketopiperazines, several other volatile compounds, including pyrrolonitrin, alcohols, sulfur containing compounds and ketones that may serve as antibiotic, bactericidal, fungicidal and pesticidal as well as agents that enhance plant productivity (Barbieri et al., 2005; Vespermann et al., 2007) were detected in different *Burkholderia* strains.

As we have seen, some of the *Burkholderia* genus has the ability to synthesize volatile compounds and these products can belong to different chemical classes such as hydrocarbons, alcohols, esters, amino, aromatic and sulfur-containing compounds. Recent studies also have demonstrated that the isolation origin of strain, pH and nutriments influence the spectrum of volatiles produced.

In this study, the objective was to report the chemical composition of microbial volatile organic compounds (MVOCs) produced by *Burkholderia* sp., a novel species belonging to the *Burkholderia* genus; a Gram negative rhizobacteria extracted for the first time from an agricultural soil samples used to grow barley and wheat soil and which is unexplored yet as a potential source of natural products for medicinal, agriculture and commercial uses. We investigated the influence of the culture medium used to culture the bacteria by comparing of the volatiles emitted by *Burkholderia* sp. using dextrose and glycerol as carbon sources. Moreover, the present article describes the extraction and the identification (using different analytical techniques GC-HRMS and derivatization reaction) of the volatile molecules from a

liquid culture broth of this strain. Different carbon sources such as glucose, fructose, starch and glycerol were used, in previous studies (Smaoui, 2010), on cultivation of bacteria and were tested for their effects on the biological activities and prior to our study, it's for the first time that we focused on the effect of carbon source on the detailed chemical composition of volatile compounds.

Material and methods

Bacteria and culture condition

Burkholderia sp. has been isolated from agricultural soil samples used to grow barley and wheat, by the dilution plating technique and their ability to solubilize Tri Calcium Phosphate (TCP). This strain has been characterized morphologically and identified by bacterial 16 S rRNA gene amplification and sequencing by PCR using the universal primer 1492 r and bacterial primer 27f. The resulting sequences were compared by BLAST search to The National Library of Medicine database (Bethesda, USA) giving an identification as *Burkholderia* sp. AGN02. The phylogenetic trees have also been established (Fig.1).

The bacterial fermentation was carried out using modified Bennett broth (Agar 15 g/L, dextrose or glycerol, peptone 2.5 g/L, pH 7.2, water 1000 mL). A single colony of *Burkolderia sp.* strain from the nutrient agar plate was inoculated into the flask containing 100 mL sterile media. The flasks were incubated in a rotatory shaker at in dark for 24h to 72h according to the needs of the bacterium. When the optical density of the culture was achieved, the bacterial mediums were then centrifuged (10,000 ×g, 20 min, 4°C) followed by filtration through a 0.22 µm filter, to obtain cell-free culture filtrate.

Chemicals used

All chemicals used were of analytical reagent grade. All reagents were purchased from Sigma-Aldrich-Fluka (Saint-Quentin France).

Extraction

The supernatant (5 L) was extracted three times with solvents of increasing polarity. Four solvents were used: cyclohexane, dichloromethane, ethyl acetate and n-butanol. The four extracts were concentrated using rotary evaporation (Rotavapor, Buchi) at 30°C to dryness to give dry mass (DM) extracts stored at -18°C.

Gas chromatography-high resolution mass spectrometry

Gas chromatography-high resolution mass spectrometry (GC-HRMS) analyses of each extracts were carried out on an HP6890 GC coupled to HRMS (GCT 1er Waters) mass selective detector fitted with an HP-5 MS fused silica capillary column (30 m, 250 µm i.d. 0.25 µm film) and piloted by Mass Lynx. For analysis: each extract (before and after derivatization) was dissolved in their solvent. One microliter of sample was injected in the split mode ratio 10. Inlet pressure was 1 kPa and helium was used as carrier gas at an on-

column flow of 1 mLmin^{-1}. The gas chromatograph was programmed as follows: 70°C to 260°C temperature rise with a gradient of 10°C/min, 12 minutes isotherm at 260°C, then a second gradient was applied to 300°C at 20°C/min and finally 16 minutes isotherm at 300°C. Total analysis time was 50 minutes. High resolution mass spectrometer was adjusted for an emission current of 663 µA and electron multiplier voltage 70 eV. Trap temperature was 250°C and that of the transfer line was 300°C.Mass scanning was performed from 40to 650 amu. The MVOCs were identified by the determination of their formula using Mass Lynx (high resolution) and by comparison with those cited in NIST 11 spectrum library. Each medium were analyzed without inoculation as negative control.

Figure 1. Phylogenetic analysis of the sequences of *Burkholderia sp.* AGN02 in comparison with their nearest relative.

Derivatization method

Using the method described by Wenclawiak et al. (1993) with some modifications, extracts were dissolved in acetonitrile in a concentration of 5 mgml^{-1}, then they were mixed with 150 µL Pierce BSTFA + 1% TMCS in a 2-mLvial. Then, the mixture was aerated with bubbling using nitrogen. The vial was closed tightly, shacked for 30 seconds and maintained at 40°C for 15 min. 1 µL of each derivatized solution was then analyzed by GC-HRMS using the same method described in previous section.

Results and discussion

All bacteria utilize the energy sources in their environment in order to use it for their maintenance and propagation. The energy sources used by different bacteria depend on the specific enzymes produced by bacteria and required for the degradation and oxidation of this carbon sources (Stanbury et al, 1995). *Burkholderia* sp. strain studied in this paper was able to produce a collection of enzymes that enable it to use dextrose and glycerol. The dextrose and glycerol were used as carbon sources to study their effect as energy sources in the production of Vocs by *Burkholderia* sp.

Chemical composition

Extraction yields

The effect of the culture medium on the yields of extracted bioactive molecules from the supernatant of *Burkholderia* sp. was investigated by the determination of the yields of extraction (Table 1). A sequential liquid/liquid extraction method was used and carried out by cyclohexane (Cyclo), dichloromethane (DCM), ethyl acetate (EtOAc) and n-butanol (BuOH). Results showed that the quantity extracted from *Burkholderia* sp. grown on dextrose was significantly highest than that obtained from the same strain grown on glycerol. Indeed, the highest quantities extracted from *Burkholderia* sp. grown on dextrose were achieved by butanol (3600 mg/L), ethyl acetate (580 mg/L), dichloromethane (62 mg/L) and the lowest was cyclohexane (18 mg/L). Similarly, for *Burkholderia* sp. grown on glycerol, the highest quantities extracted were achieved by butanol (1600 mg/L), followed by ethyl acetate (160 mg/L), dichloromethane (47 mg/L) and the lowest quantity was cyclohexane (15 mg/L). No study cited in the literature concerning extraction for this bacterium.

Table 1. Extraction quantities (mg/L) of different extracts obtained by *Burkholderia* sp.

Extraction quantities(mg/L)	Cyclohexane	Dichloromethane	Ethyl acetate	n-Butanol
Dextrose	18	62	580	3600
Glycerol	15	47	160	1600

GC-HRMS chemical composition without derivatization

No data was reported in the literature regarding the MVOCs produced by *Burkholderia* sp. This bacteria cultivated in two different culture mediums showed clear different volatile profiles (Table 2), belonging to varieties of compound classes such as saturated and unsaturated hydrocarbons, aromatic compounds, alcohols, esters, sulfur compounds, alkaloids

and diketopiperazines. For *Burkholderia* sp. grown on glycerol, no volatile compounds identified in cyclohexane and n-butanol extracts, but on dextrose, no volatile compounds identified in the butanolic extract.

Overall, 46 compounds were identified from the different extracts of the *Burkholderia* sp. strain and only four compounds were common to the two methods: 3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (*Dextrogyre* and *Levogyre*) (**18** and **22**), 3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione(*Dextrogyre*or*Levogyre*) (**34** and **36**) and octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenylpropanoate **46**. Comparatively, *Burkholderia*sp. grown on dextrosehas been able to produce long chain alkanes(C_{24} to C_{30}); eight linear alkanes were detected in the cyclohexane extract (tricosane **33**, tetracosane **37**, pentacosane **38**, hexacosane **40**, heptacosane **41**, octacosane **43**, nonacosane **44** and triacontane **45**); while the same strain grown on glycerol was unable to produce saturated hydrocarbons but it emitted two monounsaturated hydrocarbons which were present in the EtOAc extract (1-hexadecene **5** and 1-eicosene **25**).We compared our results to other strains that colonize the rhizosphere. There were no reports on the production of these saturated hydrocarbons by bacteria and fungi. Effmert et al. (2012) indicated that bacteria were generally able to produce saturated and unsaturated hydrocarbons.

Two sulfur compounds (*N*-butylbenzenesulfonamide **13** and 4,4'-thiobis(2-(*tert*-butyl)-5-methylphenol **42**) were present only in the supernatant (cyclohexane extract) of the *Burkholderia* sp. grown on dextrose. These sulfur compounds are probably the responsible for the characteristic smell of this strain (Groenhagen et al., 2013). No report on the emission of these two sulfur compounds by any species of the *Burkholderia* genus.

The highly represented class of compounds was the diketopiperazines which represent one of the major class of volatile compounds emitted by bacteria (Schulz and Dickschat 2007). In the same way as other *Burkholderia* species, the *Burkholderia* sp. used in this study produced more than one diketopiperazine. However, and prior to our study, there is no study in the literature which investigated and identified diketopiperazines in this bacterium while all diketopiperazines identified in this study were known structures. The total ion courant of all extracts and the resulting mass spectra showed eight diketopiperazines presents in the supernatant (Fig. 2). The hexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione **19**, 3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-diones (**18** and **22**), and 3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (**34** and **36**) were produced by *Burkholderia* sp. cultured in both dextrose and glycerol . The 3-methylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione **8** and 3-propylhexahydropyrrolo-[1,2-*a*]pyrazine-1,4-diones **14** and **15**

were onlyproducedby*Burkholderia*sp. cultivated on dextrose while 3-benzyl-6-methyl-2,5-piperazinedione **29**, 3-benzyl-2,5-piperazinedione **32** and 3-benzyl-6-isobutyl-2,5-piperazinedione **35** were emitted by *Burkholderia* sp. cultivated on glycerol. Three among the eight diketopiperazines were eluted with two retention times. The 3-propylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione, for example, gave two peaks (retention times, 14.59 and 14.87 min),which certify that one peak is for the *Dextrogyre* isomer and the other corresponds to the *Levogyre* isomer (Table 2).

As our culture media used to culture *Burkholderia* sp. contain yeast extract, which could lead to the production of cyclic peptide (Wang et al. 2010), it is crucial to use a negative control. The results showed the presence of a small amount of 3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-diones **18** and **22** and 3-benzylhexa-hydropyrrolo[1,2-*a*]pyrazine-1,4-dione **36**. This provides that medium used for the culture of *Burkholderia* sp. afforded also these three compounds by chemical and/or enzymatic ways. This provides evidence that these three diketopiperazines not only emitted by *Burkholderia* sp., however, it is certain that the amount of diketopiperazines formed by the bacteria is much important thanthose produced by the non-inoculated medium culture (Fig. 2). Several studies such as of Barnard and Salmon (2005) have focused on the determination of the role of this chemical class of compounds in Gram-negative bacteria, and since 2005, diketopiperazines were classified as novel signal molecules of this type of bacteria (Barnard and Salmon 2005).

Two others alkaloids *N,N*-dimethyldodecan-1-amine **2** and *N,N*-dimethyltetradecan-1-amine**7**were identified only in the cyclohexane extract made from *Burkholderia* sp. grown on dextrose.

Furthermore, *Burkholderia*sp. grown on dextrose showed the ability to produce a plethora of esters. Several esters like, methyl palmitate, dibutylphtalate, (*E*)-methyl octadec-9-enoate**27** and methyl 14-methylheptadecanoate,, while *Burkholderia* sp. grown on glycerol was able to produce only two other esters in addition to octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate **46**, which was common for the two culture methods. However, the detection of glycerol **1** and luteolin **11** in the EtOAc extract of *Burkholderia* sp. cultured in glycerol let us to suppose that the latter may be esterified by three units of oleic acid by this strain cultured in a specific condition and via an enzymatic way. We compared our results to other species that colonize the rhizosphere. There were no reports on the production of these esters from this Gram-negative strain. Silvia et al. (2013) indicates that, generally, bacteria especially *Burkholderia tropica* were able to produce esters.

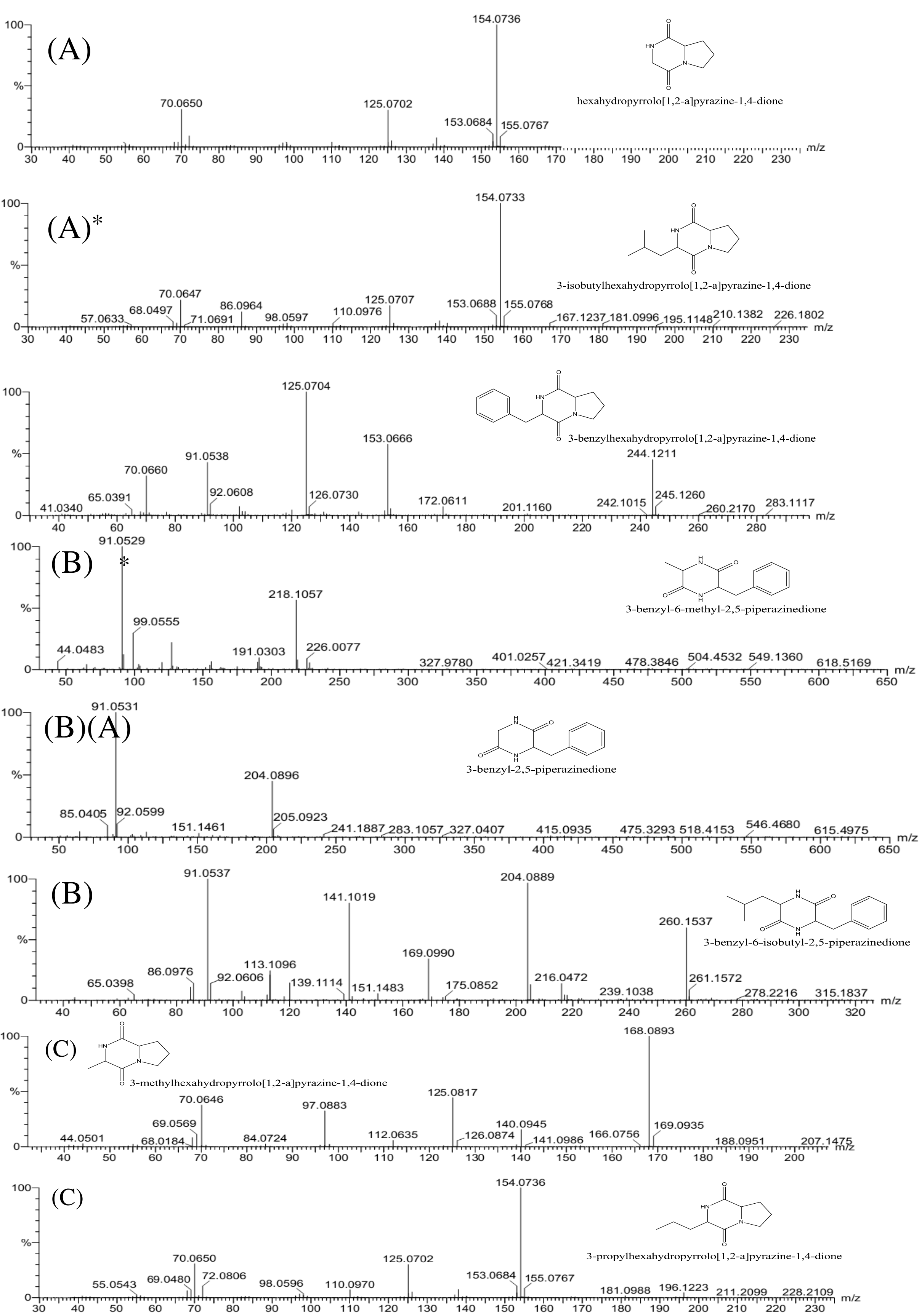

Figure 2. EI-HRMS of diketopiperazines detected in different extracts of the *Burkholderia*sp. (a) common; (b) glycerol; (c) dextrose; *: present in unoculated medium.

OH
OH
OH
glycerol **1**
+
O
OH
oleic acid
Enzymatic way
O
O
O
O
O
O
triolein **11**

Figure 3. Mechanism of the formation of triolein **11.**

From the above data, it is important to notice that when grown on dextrose *Burkholderia* sp. was able to produce phthalate derivatives like diisobutyl phthalate **16** and bis(2-ethylhexyl) phthalate **39**) detected in the corresponding cyclohexane extract; while when grown on glycerol, it was able to produce only one, the diethyl phthalate **6** detected in the dichloromethane extract and not detected when dextrose is used as a carbon source. Comparatively to other species that colonize the rhizosphere, these phthalate derivatives and other derivatives have been previously isolated from bacterial culture broths (Smaoui, 2010).
Amazingly, *Burkholderia* sp. grown on glycerol showed the ability to produce phenolic derivatives and aliphatic alcohols such as 3-ethoxy-4-methoxyphenol and 1-octadecanol detected in the corresponding ethyl acetate extract while the same strain grown on dextrose was unable to produce these classes of compounds. Also, it is important to notice that the 2,4-di-*tert*-butylphenol might result from the enzymatic hydrolysis of 2,4-di-*tert*-butylphenyl 5-hydroxypentanoate. The comparison of our results to other Gram negative bacteria showed that these alcohol compounds were produced for the first time by our *Burkholderia* species. Kanchiswamy et al. (2015) indicated that alcohols represent one of the major class of volatile compounds emitted by bacteria.

Table 2. GC-HRMS analysis of VOCs produced by *Burkholderia* sp. without derivatization.

N°	Rt (min)	Compound	*Structures*	*Burkholderia*sp.							
				dextrose				glycerol			
				cyclo	DCM	AcOEt	BuOH	cyclo	DCM	AcOEt	BuOH
1	4.50	glycerol								×*	
2	10.96	*N,N*-dimethyldodecan-1-amine		×							
3	10.99	2,4-di-*tert*-butylphenol								×	
4	11.01	2,4-di-*tert*-butylphenyl 5-hydroxypentanoate		×							
5	11.86	hexadecane								×	
6	11.92	diethyl phthalate							×		

7	13.31	*N,N*-dimethyltetradecan-1-amine		×				
8	13.47	3-methylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione			×			
9	13.53	3-ethoxy-4-methoxyphenol					×	
10	13.64	2-(2-aminopropanamido)-4-methylpentanoic acid				×		
11	13.95	triolein						×
12	14.12	1-octadecanol						×
13	14.25	*N*-butylbenzenesulfonamide		×				
14	14.59	3-propylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione(dextro or levo)			×	×		

15	14.87	3-propylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione(dextro or levo)			×					
16	15.00	diisobutyl phthalate		×						
17	15.47	7,9-di-*tert*-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione		×						
18	15.56	3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione(dextro or levo)		×	×*	×*			×*	
19	15.57	hexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione								×
20	15.64	methyl palmitate		×						

21	15.70	methyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate		×						
22	15.76	3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione(dextro or levo)			×*				×*	×
23	15.84	hexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione				×				
24	15.97	dibutyl phthalate		×						
25	16.18	1-eicosene								×
26	16.45	*N*-benzyl-2-phenylacetamide		×						
27	17.39	(*E*)-methyl octadec-9-enoate		×						
28	17.57	methyl 14-methylheptadecanoate		×						

29	17.91	3-benzyl-6-methyl-2,5-piperazinedione								×
30	18.01	dodecyl octanoate		×						
31	18.06	1-tricosanol								×
32	18.18	3-benzyl-2,5-piperazinedione								×
33	19.11	tricosane	$C_{23}H_{48}$	×						
34	19.55	3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione(dextro or levo)							×	×
35	19.60	3-benzyl-6-isobutyl-2,5-piperazinedione								×
36	19.86	3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione(dextro or levo)			×*					×*
37	19.94	tetracosane	$C_{24}H_{50}$	×						
38	20.82	pentacosane	$C_{25}H_{52}$	×						

39	21.12	bis(2-ethylhexyl) phthalate		×	
40	21.85	hexacosane	$C_{26}H_{54}$	×	
41	23.10	heptacosane	$C_{27}H_{56}$	×	
42	23.99	4,4'-thiobis(2-(*tert*-butyl)-5-methylphenol)		×	
43	24.65	octacosane	$C_{28}H_{58}$	×	
44	26.60	nonacosane	$C_{29}H_{60}$	×	
45	29.09	triacontane	$C_{30}H_{62}$	×	
46	39.97	octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenylpropanoate		×	×

*: present in non-inoculated culture media

GC-HRMS chemical composition after derivatization

We should notice that no data have been reported in the literature regarding the derivatization reaction of different extracts from *Burkholderia* sp. strain cultivated in two different culture media. The results showed a significant difference between the two derivatized volatile profiles (Table 3) due to a diversity of labile hydrogen compounds comprise such as alcohols, phenols, amines and carboxylic acids. Our findings did not show any derivative compounds identified in the cyclohexane extracts of *Burkholderia* sp. grown either on dextrose or on glycerol.

On the other hand, overall, 21 compounds were identified from the different extracts of *Burkholderia* sp. after derivatization and only 3 common compounds were emitted by this strain grown in dextrose and glycerol: glycerol **1**, butan-2,3-diol **53** and 3-hydroxybutanoic acid **56**. *Burkholderia* sp. grown on dextrose showed the ability to produce several carboxylic acids like 2-methylbutanoic acid **49**, palmitic acid and 3,4,5-trihydroxypentanoic acid **60** . However, the same strain grown on glycerol was able to produce only the 3-hydroxybutanoic acid **56** which was common for the two carbon sources used. These carboxylic acids were commonly known to be emitted by bacteria and fungi, but reported for the first time from *Burkholderia* sp.

Beside carboxylic acids, two compounds containing a furan moiety identified as 5-(1,2-dihydroxyethyl)-4-hydroxydihydrofuran-2(3*H*)-one **64** and 1-(3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)pyrimidine-2,4(1*H*,3*H*)-dione **66**) were detected only in the BuOH extract of the *Burkholderia* sp. grown on dextrose. This class of compounds is known to be responsible of the induction of bacterial response, which led us to notice that *Burkholderia* sp. grown on dextrose may induce bacterial response unlike to *Burkholderia* sp. grown on glycerol. To our knowledge there were no report for production of furan containing compounds from *Burkholderia* sp.

The most abundant compound in both strains was butan-2,3-diol **53**. Beside this diol, several other alcohols that included ethan-1,2-diol **51**, pentan-2-ol **54** as well as butan-1,2,3-triol **57** and butan-1,2,3,4-tetraol **61** were present in *Burkholderia* sp., grown on dextrose, extracts. This class of compounds was also present in the *Burkholderia* sp., grown on glycerol, extracts while they were less abundant. Indeed, beside butan-2,3-diol **53** and glycerol **1**, two other alcohols including propan-1,2-diol **52** and propan-2-ol **58** were present. Alcohols were known to interfere with fungi (Kanchiswamy et al., 2015). The abundance of alcohol compounds in different *Burkholderia* sp., grown on dextrose, extracts led as to suppose that this strain may be possessed a good anti-fungal activity that we will evaluate in a future studies.

Table 3. GC-HRMS analysis after derivatisation.

N°	Rt (min)	Compound	Structure	*Burkholderia*sp.							
				Dextrose				glycerol			
				cyclo	DCM	EtOAc	BuOH	cyclo	DCM	EtOAc	BuOH
47	5.71	3-hydroxy-2-butanone(dextro or levo)			×						
48	6.26	3-hydroxybutan-2-yl acetate				×					
49	6.47	2-methylbutanoic acid			×						
50	6.71	3-methylbutanoic acid			×						
51	7.82	ethan-1,2-diol					×				
52	7.99	propan-1,2-diol								×	
53	8.53	butan-2,3-diol			×	×	×		×		×

54	9.43	pentan-2-ol		×						
55	9.65	3-hydroxy-2-butanone (dextro or levo)		×						
56	10.22	3-hydroxybutanoic acid		×		×			×	×
57	10.69	butan-1,2,3-triol			×					
1	11.45	glycerol		×		×		×	×	×
58	11.90	propan-2-ol							×	
59	12.26	octanoic acid		×						
60	12.45	3,4,5-trihydroxypentanoic acid				×				
61	13.61	butan-1,2,3,4-tetraol				×				

62	15.12	benzo[*d*]oxazol-2-amine				×
63	16.07	testosterone				×
64	17.33	5-(1,2-dihydroxyethyl)-4-hydroxydihydrofuran-2(3*H*)-one			×	
65	17.52	palmitic acid		×		
66	20.34	1-(3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1*H*,3*H*)-dione			×	

Moreover, only one ketone produced in its two forms *levogyre* and *dextrogyre* (3-hydroxy-2-butanone **47** and **55**) was presented and only in *Burkholderia* sp. grown on dextrose. This compound was widely present in bacteria and known for its stimulation of plant growth (Ryu et al., 2004; Rudrappa et al., 2010).

Beside these classes of compounds, *Burkholderia* sp. grown on glycerol was found to be able to produce steroid (testosterone) **63** and benzo[*d*]oxazol-2-amine **62** detected in the EtOAc extract, while, the same strain grown on dextrose was unable to produce these classes of compounds but it afforded the only ester (3-hydroxybutan-2-yl acetate) **48** detected on its EtOAc extract. We should notice that these three compounds were identified for the first time in this strain.

Our work highlighted the significant difference in chemical composition of *Burkholderia* sp. between the two mediums containing, cultivated on dextrose and glycerol. Both strains were able to produce different diketopiperazines (Fig. 4) known for their important role as a signal molecule of Gram-negative bacteria and may also play a key role in cross-talk of bacteria.

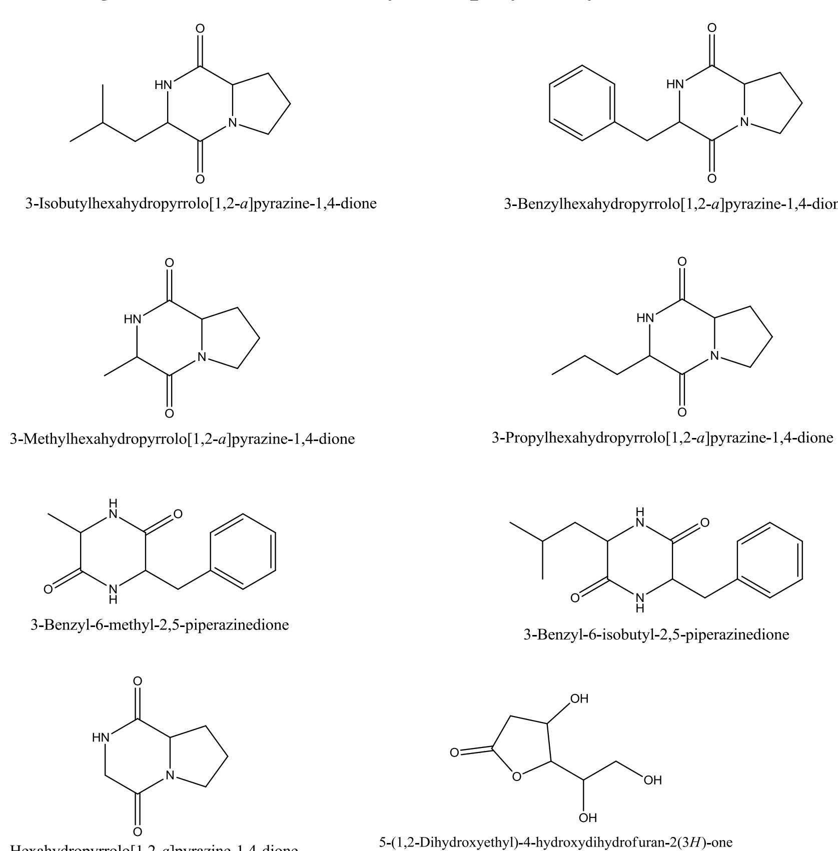

Figure 4. Signal molecules secreted by Burkholderiasp.: volatiles auto-inducers of the quorum-sensing systems.

Only *Burkholderia* sp. grown on dextrose was found to be able to produce another class of molecules (5-(1,2-dihydroxyethyl)-4-hydroxydihydrofuran-2(3*H*)-one) also used as signal molecule (Fig. 4) (Wang et al. 2010). It is important to notice that a great difference was observed in the chemical structure of diketopiperazines identified from both strains which led us to suppose that both strain will interfere differently with external organisms. In addition, the secretion of furan containing compounds by *Burkholderia* sp. grown on dextrose only, confirm our hypothesis.

The production of diketopiperazines, known as signal molecule of Gram-negative bacteria, and furan containing compounds known to induce bacterial responses depends on the nature of carbon sources.

Moreover, in the presence of dextrose or glycerol as carbon sources *Burkholderia* sp. was able to produce volatile compounds known as plant defense and growth modulators (Ryu et al., 2004; Rudrappa et al., 2010; Zou et al., 2010), we cite here butan-2,3-diol **53** as a common alcohol (dextrose and glycerol)and5-(1,2-dihydroxyethyl)-4-hydroxydihydrofuran-2(3*H*)-one **64**, 1-(3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)pyrimidine-2,4(1*H*,3*H*)-dione **66** and 3-hydroxy-2-butanone (*levogyre* and *dextrogyre* forms) **47** and **55** which were only produced by *Burkholderia* sp. grown on dextrose (Fig. 5). These results clearly demonstrate the influence of the nature of carbon sources on the production of plant defense and growth modulators MVOCs by both strains. We now suppose that MVOCs as plant defense and growth modulators is preferentially produced by *Burkholderia* sp. grown on dextrose.

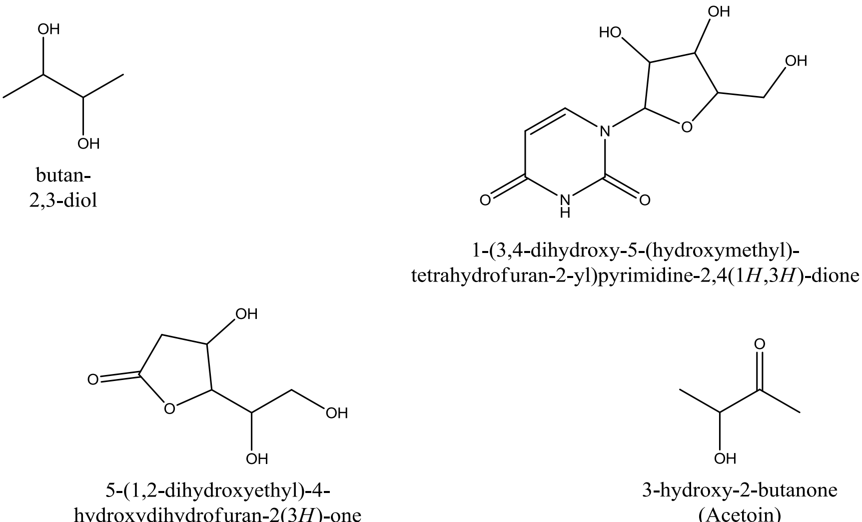

Figure 5. Volatiles able to induce plant response.

A significant difference was observed between the volatile profiles from *Burkholderia* sp. grown on dextrose when compared with those from *Burkholderia* sp. grown on glycerol, suggesting that carbon sources play a major role in the production of volatiles.

Over the last decade, microbial volatile emissions and differentiation according to their environmental niches have received increasing attention. This question was assessed in our study by using two different culture medium; dextrose and glycerol; *Burkholderia* sp. was used as a model species. And to the best of our knowledge, studies focused on the effect of the carbon sources used to culture *Burkholderia* sp. have not been reported previously. We identified compounds from a huge variety of substance classes, although, remarkable differences in the volatile emission were observed, especially in the production of signal molecules such as diketopiperazines which was remarkably influenced, indicating that response of *Burkholderia* sp. to external organisms will be different.

Conclusion

The aim of this study was to determine whether volatile emission of a *Burkholderia* strain would be influenced by the use of different carbon sources. Our results indicate the presence of different classes of compounds such as hydrocarbons, alcohols, ketones, esters, carboxylic acids and diketopiperazines. We identified and characterized several diketopiperazines such as 3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (**18** and **22**) and 3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (**34** and **36**) known for their intervention in bacterial-bacterial and bacterial-plant interactions. It is regrettable that this work could not clarify the role of each identified compounds as well as their possible potential for plant growth. Thus, further studies are necessary for testing the bioactivity of these extracts issue from *Burkholderia* sp. by exploring the action of extracts and single pure compounds which may be promising candidates not only for agriculture applications but also for pharmaceutical uses.

References

Barbieri, E., Gioacchini, A.M., Zambonelli, A., Bertini, L., Stocchi V., 2005. Determination of microbial volatile organic compounds from *Staphylococcus pasteuri* against *Tuber borchii* using solid-phase microextraction and gas chromatography/ion trap mass spectrometry. Rapid Commun. Mass Spectrom. 19, 3411-3415.

Barnard, A.M.L., Salmond G.P.C., 2004. Quorum sensing: the complexities of chemical communication between bacteria. Complexus 5, 87-101.

Bartolucci, G., 2014. GC-MS volatolomic approach to study the antimicrobial activity of the antarctic bacterium *Pseudoalteromonas sp* TB41. Metabolomic 10, 42-51.

Blom, D., Fabbri, C., Connor, E., Schiestl, F., Klauser, D., Boller, T., et al. 2011. Production of plant growth modulating volatiles is widespread among rhizosphere bacteria and strongly depends on culture conditions. Environ. Microbiol. 13, 3047-3058.

Effmert, U., Kalderás, J., Warnke, R., Piechulla, B., 2012. Volatile mediated interactions between bacteria and fungi in the soil. J. Chem. Ecol. 38, 665-703

El-Banna, N., Winkelmann, G., 1998. Pyrrolnitrin from *Burkholderia cepacia*: antibiotic activity against fungi and novel activities against streptomycetes. J. Appl. Microbiol. 85, 69-78.

Groenhagen, U., Baumgartner, R., Bailly, A., Gardiner, A., Eberl, L., Schulz, S., Weisskopf L., 2013. Production of bioactive volatiles by different *Burkholderia ambifaria* strains. J. Chem. Ecol. 39, 892-906.

Kanchiswamy, C.N., Malnoy, M., Maffei, M.E., 2015. Chemical diversity of microbial volatiles and their potential for plant growth and productivity. Front Plant Sci. 6, 1-23.

Mahenthiralingam, E., Song, L., Sass, A., White, J., Wilmot, C., Marchbank, A., Boaisha, O., Paine, J., Knight, D., Challis, G.L., 2011. Enacyloxins are products of an unusual hybrid modular polyketide synthase encoded by a cryptic *Burkholderia ambifaria* Genomic Island. Chem. Bio. 18, 665–677.

Mitchell, R.E., Frey, E.J., Benn, M.K., 1986. Rhizobitoxine and 1-threohydroxythreonine production by the plant pathogen *Pseudomonas andropogonis*. Phytochem. 25, 2711-2715.

Moebius, N., Ross, C., Scherlach, K., Rohm, B., Roth, M., Hertweck, C., 2012. Biosynthesis of the respiratory toxin bongkrekic acid in the pathogenic bacterium *Burkholderia gladioli*. Chem. Biol. 19, 1164–1174.

Rudrappa, T., Biedrzycki, M.L., Kunjeti, S.G., Donofrio, N.M., Czymmek, K.J., Pare P.W., 2010. The rhizobacterial elicitor acetoin induces systemic resistance in *Arabidopsis thaliana*. Commun. Integr. Biol. 3, 130-138.

Ryu, C.M., Farag, M.A., Hu C.H., Reddy M.S., Kloepper J.W., Pare P.W., 2004.Bacterial volatiles induce systemic resistance in Arabidopsis. Plant Physiol. 134, 1017-1026.

Pidot, S.P., Coyne, S., Kloss, F., Hertweck, C., 2014. Antibiotics from neglected bacterial sources. Intern. J. Med. Microbiol. 304, 14-22.

Schulz, S., Dickschat, J.S., 2007. Bacterial volatiles: the smell of small organisms. Nat. Prod. Rep. 24, 814-842.

Silvia, T.S., Raunel, T., Rafael, V.D., Jesus, C.M., Ernesto, P.R., 2013. Identification of volatile compounds produced by the bacterium *Burkholderia tropica* that inhibit the growth of fungal pathogens. Bioengineered 4, 236-243.

Smaoui, S., 2010. Purification and Characterization of biomolecules from microorganisms recently isolated and identified. *PhD thesis. Toulouse III University - Paul Sabatier.*

Stanbury, P.F., Whitaker, A., Hall, S.J., 1995. Media for industrial fermentations. Principles of Fermentation Technology 2nd ed., Paris, pp. 93-121.

Vespermann, A., Kai, M., Piechulla, B., 2007. Rhizobacterial volatiles affect the growth of fungi and *Arabidopsis thaliana*. Appl. Environl. Microbiol. 73, 5639-5641.

Vial, L. Lépine, F., Milot, S., Groleau, M.C., Dekimpe, V., Woods, D.E., Déziel, E., 2008. *Burkholderia pseudomallei, B. thailandensis,* and *B. ambifaria* produce 4-hydroxy-2-alkylquinoline analogues with a methyl group at the 3 position that is required for quorum-sensing regulation. J. Bacteriol. 190, 5339-5352.

Wang, J.-H., Quan, C.-S., Qi, X.-H., Li, X., Fan, S.-D., 2010. Determination of diketopiperazines of *Burkholderia cepacia* CF-66 by gas chromatography-mass spectrometry. Anal. Bioanal. Chem. 396, 1773-1779.

Wenclawiak, B.W., Jensen, T.E., Richert, J.F.O., 1993. GC-MS-FID analysis of BSTFA derivatized polar components of diesel particulate matter (NBS SRM 1650) extract. Fresenius J. Anal. Chem. 6, 808-812.

Zou, C., Li Z., Yu, D., 2010. *Bacillus megaterium* strain XTBG34 promotes plant growth by producing 2-Pentylfuran. J. Microbiol. 48, 460-466.

Partie B: Chemical composition, pharmaceutical and allelopathic activities of a new *Burkholderia* sp. AGN02 strain extracts

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Abstract

The purpose of the present work was to report the effect of carbon sources (dextrose and glycerol) used on the cultivation of new *Burkholderia* specie on the chemical composition and biological activities. Supernatants of *Burkholderia* sp. AGN02 were extracted with different organic solvents (cyclohexane, dichloromethane, ethyl acetate and butanol) and extracts were analyzed for their total phenolics, anti-5-lipoxygenase, anti-acetylcholinesterase, anti-xanthine oxidase, anti-α-amylase, cytotoxic activities (HCT116, MCF-7, IGROV and OVCAR cell lines) and also their potential allelophatic activities assessed on maize and sunflower seeds.

Results showed that the substitution of dextrose by glycerol significantly affected the total phenolic amount and increased the antidiabetic (62.7% inhibition for ethyl acetate extract at 50 µg/mL), anti-acetylcholinesterase and anticancer activities, unlike anti-5-lipoxygenase and allelopathic activities that reduce. Ethyl acetate extract grown on dextrose exhibited the highest allelopathic potential (97.92% germination at 200 µg/mL). It was found that the cyclohexane extract obtained from medium grown on glycerol possessed the most evident cytotoxic activity at 50 µg/mL against HCT-116, MCF-7, IGROV and OVCAR cells (63.0, 48.3, 48.2 and 22.2%, respectively).Results allowed us to conclude that the nature carbon source influenced considerably the chemical composition content and the biological activities of *Burkholderia*.

Keywords: *Burkholderia* sp.; anti-acetylcholinesterase; anti-xanthine oxidase; antidiabetic; cytotoxic; allelopathic.

Introduction

The *Burkholderia* family, known as a β subdivision of the proteobacteria, is a large family that comprises more than forty species inhabiting large variety of niches such as soil, plant rizophere, water, hospital environment, animals and humans. Several species of this family have been known as plant pathogens such as *B. caryophylli*, *B. glumae* and *B. cepacia* (Vial *et al.*, 2007) and also as human pathogens such as *B. pseudomallei B. gladioli*, *B. fungorum* and *B. mallei* (De Soyza *et al.*, 2008).

In contrast, *Burkholderia* species are also known to produce a wide range of volatile and non-volatile secondary metabolites suchas pyrrolnitrin, diketopiperazine, ketones, sulfur containing compounds, phenazine and other unidentified compounds (Groenhagen *et al.*, 2013; Wang *et al.*, 2010). Moreover *Burkholderia* species have beenused since ancient times in agricultural practice (Kanchiswamy *et al.*, 2015), antifungal (Elshafie *et al.*, 2012) agents that increases the crop yield, (Blom *et al.*, 2011; Kanchiswamy *et al.*, 2015) antibiotic and antibacterial (Hwang, *et al.*, 2002).

The purpose of the present study was to determine and compare properties of new *Burkholderia* sp., *Gram* negative specie. We report here the effect of carbon sources (dextrose and glycerol) used on the cultivation of this bacterium on the chemical composition and biological activities. Supernatants were extracted with different organic solvents (cyclohexane, dichloromethane, ethyl acetate and butanol) and extracts were analyzed for total content of phenolics, anti-inflammatory (anti-5-lipoxygenase), anti-acetylcholinesterase, anti-xanthine oxidase, antidiabetic (anti-α-amylase), cytotoxic activities (HCT116, MCF-7, IGROV and OVCAR cell lines) and also their potential allelophatic activities assessed on maize and sunflower seeds. To the best of our knowledge, no biological study has been performed on this *Burkholderia* strain.

Materials and methods

Chemicals

All chemicals used were of analytical reagent grade. All reagents were purchased from Sigma-Aldrich-Fluka (Saint-Quentin, France).

Studied bacterial strain

This *Burkholderia* sp. has been isolated from agricultural soil samples used to grow barley and wheat, by the dilution plating technique and their ability to solubilize TriCalcium Phosphate. This strain has been characterized morphologically and identified by bacterial 16 S rRNA gene amplification and sequencing by PCR using the universal primer 1492 r and bacterial primer 27f. The resulting sequences were compared by BLAST search to The National Library of Medicine database (Bethesda, USA) giving an identification as *Burkholderia* sp. AGN02. The phylogenetic trees have also been established The *Burkholderia* sp. AGN02 strain was grown in a modified Bennet medium with dextrose or glycerol (10 g) as carbon source, yeast extract (1.5 g), peptone (2.5 g) instead of meat extract, initial pH is adjusted to 7.2±0.2.

Extraction

For extraction, five liters of each filtered supernatant were extracted with ten liters, successively, by different solvents of increasing polarity: cyclohexane, dichloromethane, ethyl acetate and n-butanol. All organic extracts were evaporated and concentrated by rotary evaporation under vacuum at 30°C.

Total phenolic amount

The total phenolic content of the different obtained extracts was assessed by spectrometry using the “Folin-Ciocalteu” reagent assay (Bekir *et al.*, 2013). In a 96-wellplate, 20 µL of the diluted extract solution, prepared at a concentration of 3mg/mL, was mixed with 100 µL of Folin-Ciocalteu reagent (0.2 mol/L). The mixture was then agitated for 30sec and rested for 5 min in the darkness. 80 µL of sodium carbonate solution diluted with distilled water (75g/L) was added and the mixture was agitated. After incubation during 15 min, the absorbance was measured at 765 nm. Gallic acid, a standard for the calibration curve, was used at concentration ranging from 0 to 30 mg/L. The result of quantification was expressed as mg of gallic acid equivalent per gram of dry mass (mg GAE/g DM).

Anti-5-lipoxygenanse assay

Anti-inflammatory evaluation was performed using the method described by Bekir *et al.* (2013). Various extracts were tested at the concentration of 50 µg/mL in 96-well plates incubated at 25°C for 10 min. Each well of 96-well plate contained a total volume of 250 µL (150 µL phosphate buffer (pH 7.4), 20 µL sample, 60 µL of linoleic acid (3.5 mmol/L) and 20 µL 5-lipoxygenase (soybean 500 U) and the absorbance was measured at 234 nm in a Multiskan Go spectrophotometer. The activity was defined as the percentage of inhibition of the 5-lipoxygenase enzyme. The nordihydroguaiaretic acid (NDGA) was used as standard.

Anti-acetylcholinesterase assay

The anti-acetylcholinesterase activity of extracts was determined by the detection of the 5-thio-2-nitrobenzoic acid, yellow product, formed after the action of thiocholine on DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) using a spectrophotometer at 412 nm. The method established by Kammoun El Euch et al., (2015) was used with some modifications. 25 µL of extracts (500 µg/mL), 50 µL of phosphate buffer (pH 8), 125 µL of DTNB and 25 µL of the acetylcholinesterase enzyme (AChE) were added in wells of 96-well plates. After 15 min pre-incubation at 25°C, 25 µL of acetylcholine iodide was added and the absorbance was measured at 412 nm after 10 min incubation. Similarly, the control was prepared using the same procedure expects that extract was replaced by 5% dimethyl sulfoxide (DMSO) and positive control was prepared using the same procedure replacing sample with galanthamine.

Anti-xanthine oxidase assay

The xanthine oxidase is an enzyme that catalyse the formation of oxygen radical species and uric acid (Kammoun El Euch *et al.*, 2015). 60 µL of sodium phosphate buffer (pH 7.5), 30 µL of xanthine oxidase (0.1 µmol/L) and 50 µL of extract (200 mg/L) were placed in wells of 96-well plates. After 15 min pre-incubation at 25°C, 60 µL of xanthine was added and was incubated for 10 min. The control was prepared using the same procedure except that the extract with5% DMSO solution. Then the absorbance was measured at 295 nm and the activity was measured as a percentage of inhibition of the xanthine oxidase. Allopurinol, known as a potent anti-xanthine oxidase was used as reference.

Anti-α-amylaseassay

The α-amylase inhibitory assay was carried out using the procedure of Shalaby et al., (2014) with some modifications. 50 µL of extracts (1.3 mg/mL) was placed in a tube and 50 µL of α-amylase solution (0.58 mg/mL) was added. After 10 min pre-incubation at 25°C, 100 µL of 1% of starch solution was added to the content of the tube and was incubated for 3 min at 25°C. Then, 100 µL of dinitrosalicylic acid reagent was added. Finally and after 10 min incubation in boiling water (100°C), the mixture was diluted with 1mL sodium phosphate buffer (pH 6.9) and the absorbance measured at 530 nm in a spectrophotometer. The control was prepared using the same procedure by replacing the extract with H_2O/DMSO. Acarbose was used as standard. The activity was calculated as a percentage of inhibition of the α-amylase.

Cytotoxic assay

Extracts were tested for their anticancer activity against the following cells lines: human colon cancer (HCT-116), human breast cancer (MCF-7) and two human ovarian cancers (IGROV and OVAR). MCF7 cell was maintained in DMEM growth medium and the three other cells lines were maintained in RPMI growth medium. Cytotoxic effect was determined using a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method described by Bekir *et al.* (2013) In 96-well plates, we placed 100 µL of culture medium containing 10^4 cells/well. After 24h incubation at 37°C, we added 100 µL of culture medium containing samples such that the final sample concentration was 50 µg/mL per well and then, the mixture was incubated at 37°C for 48h in a humidified atmosphere with 5% CO_2.

The mixture was then aspirated and replaced with 50 µL of 1 mg/mL MTT and incubated for a further 40 min at 37°C. The MTT solution was then replaced with 50 µL of DMSO to dissolve the MTT formazan precipitate and the absorbance was read at 605 nm on a MULTISKAN GO spectrophotometer. The cytotoxic effect of the different extracts was estimated in terms of growth inhibition percentage. Doxorubicin and tamoxifen were used as references.

Allelopathic potential

Each extract was tested on maize (*Zea mays* L.) and sunflower (*Helianthus annuus* L.). Seeds were surface-sterilized with sodium hypochlorite (10%) for 6 min, then rinsed three times with physiologic water (NaCl 0.9%). Three replicates, each comprising twenty four imbibed seeds of maize and sunflower were separately placed on the supporting medium in sterilized

multiwall dishes; 50 µL of each extract at a concentration of 200 µgmL^{-1} were applied for each treated seed. Imbibed seeds were used to evaluate the effect of each extracts on germination. Percentage of seed germination was determined by counting the number of seeds that had germinated after 9 days for maize and 13 days for sunflower.

The index of germination (GI), which represents the delay in germination induced by the different extracts, was calculated using the following equation (Omezzine *et al.*, 2013):

$GI=(N_1) \times 1 + (N_2-N_1) \times 1/2 + ... + (N_n-N_{n-1}) \times 1/n$

Where N_n is the proportion of germinated seeds observed after n days.

Statistical Analysis

All data were expressed as means ± standard deviations of three triplicate measurements. Differences between the means were established using ANOVA test and standard deviations (S.D.) did not exceed 5% for the majority of the obtained values.

Results and discussion

Extraction

Different extracts were obtained from the two supernatants of *Burkholderia* sp. by using a sequential liquid/liquid method constituted by four solvents: cyclohexane, dichloromethane, ethyl acetate and n-butanol. The extraction quantities of secondary metabolites of *Burkholderia* strain were cited in Figure 1. Detailed results showed that extractions made from *Burkholderia* sp. grown on dextrose provided higher yield than from *Burkholderia* sp. grown on glycerol. The highest quantities extracted from the *Burkholderia* sp. grown on dextrose were provided by n-butanol (3600 mg/L) followed by ethyl acetate (580 mg/L) then dichloromethane (62 mg/L) and the lowest amount obtained was with cyclohexane (18 mg/L). Similarly, the highest quantities extracted from *Burkholderia* sp. grown on glycerol, were achieved by n-butanol (1600 mg/L), followed by ethyl acetate (160 mg/L), dichloromethane (47 mg/L) and the lowest quantity was also given by cyclohexane (15 mg/L).We should notice that the variation in the quantities of different extracts was attributed to the polarity of the secondary metabolites present in *Burkholderia* sp.

Total phenolic content

This is the first study which recorded the phenolic content of extracts from *Burkholderia* sp. The total phenolic contents in the different *Burkholderia* extracts were cited in Figure 1. Results showed that the amount of total phenolics varied in the different extracts and ranged

from 3.0±0.1 to 21.1±1.3 mg GAE/g DM. The highest amount of phenolic compounds was recorded for glycerol n-butanol extract (21.1±1.3 mg/g GAE DM) of *Burkholderia* sp. grown on glycerol. The ethyl acetate, dichloromethane and cyclohexane extracts of the latter were found to be less rich in these compounds (6.4±0.3, 4.0±0.4 and 3.7±0.4 mg/g GAE DM, respectively). This finding allowed us to note that most phenolic compounds produced by this strain when grown on glycerol are relatively polar. In contrast, for the *Burkholderia* sp. grown on dextrose, n-butanol extract was found less rich in phenols (3.0±0.1 mg GAE/g DM). This finding shows clearly the importance of the nature of the carbon source to produce such compounds with specific structure and polarity. The highest amount of phenolics was recorded in the dichloromethane extract (7.6±0.2 mg GAE/g DM), followed by ethyl acetate extract (4.7±0.1 mg GAE/g DM) and cyclohexane extract (3.7±0.1 mg GAE/g DM).

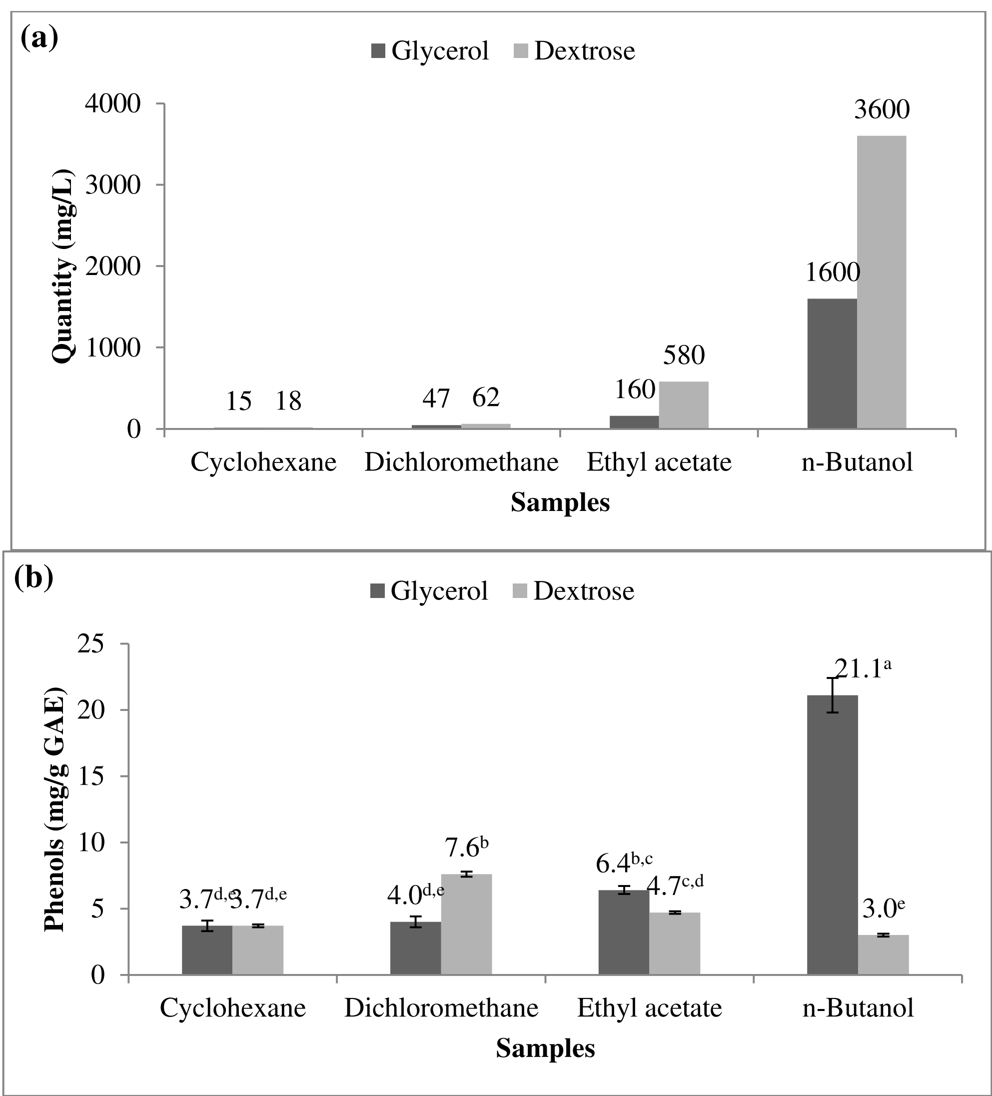

Figure 1. Extraction quantities (a) and total phenolic content (b) of different extracts from *Burkholderia* sp. AGN02 grown on dextrose and glycerol.

5-lipoxygenase inhibitory activity

The 5-lipoxygenase inhibitory potentials by different extracts of *Burkholderia*, tested at 50µg/mL, werepresented in Table 1. Different extracts obtained from *Burkholderia* grown on dextrose exhibited 5-lipoxygenase inhibition, with percent inhibition values ranged from 12.8±0.3 to 18.9±0.4%, however the different extracts obtained from the same strain grown on glycerol were found to be unable to inhibit the 5-lipoxygenase. These results showed that the secretion of potent molecules by *Burkholderia* which may exhibit anti-inflammatory activity was affected by the nature of carbon sources. To our knowledge, no study of the anti-inflammatory activity of *Burkholderia* sp. or other *Burkholderia* species was cited in the literature.

Anti-acetylcholinesterase (AChE) activity

The results given in Table 1 showed that all extracts from *Burkholderia* grown on glycerol were able to inhibit the AChE, with percent inhibition values ranging from 14.7±2.7% to 23.7±4.2% at 50 µg/mL. On the other hand, only the butanolic extract from *Burkholderia* grown on dextrose showed anti-AChE activity (14.9±0.8%). We can conclude that glycerol, used as carbon source, has allowed the secretion of bioactive molecules against acetylcholinesterase enzyme which were successfully extractable using different solvents. Also, the nature (structure and polarity) of compounds extracted by n-butanol from the same strain grown on dextrose were able to inhibit this enzyme. In conclusion, the nature of the carbon source used in the culture medium of our studied strain has directly affected the secretion of bioactive molecules involved in the inhibition of acetylcholinesterase. To our knowledge, the anti-AChE potential of extracts from *Burkholderia* sp. were reported here for the first time.

Anti-xanthine oxidase activity

The anti-xanthine oxidase activities were assessed for the first time for all extracts of *Burkholderia* sp by the determination of the percent of inhibition at 50 µg/mL. From the results presented in Table 1, we observed that all extracts, except the inactive cyclohexane extract made from *Burkholderia* sp. grown on dextrose showed on xanthine oxidase ranging from 6.4±0.5 to 14.0±1.1%, with the exception of the cyclohexane extract which was found to be inactive at the used concentration. The inhibition percentages obtained in this study by *Burkholderia* sp. extracts grown on glycerol were slightly higher than those of the same strain grown on dextrose. Their percentage inhibition ranged from 9.3±1.5 to 19.7±2.6%. The ethyl

acetate extract displayed the highest activity (19.7±2.6%). The comparison of the activity of the same extract obtained from this strain grown on the two carbon sources shows the contribution of the latter to produce compounds with specific structure in each case to induce such activity.

Table 1. Anti-5-lipoxygenase, anti-acetylcholinesterase and anti-xanthine oxidase activities of *Burkholderia* sp. extracts. Results are expressed as percentage of enzyme inhibition at 50 µg/mL.

Extract		Anti-5-lipoxygenase activity	Anti-acetylcholinesterase activity	Anti-xanthine oxidase activity	anti -α-amylase activity
***Burkholderia* grown on dextrose**	Cyclohexane	12.8±0.3[d]	na	na	23.0±1.8[c,d]
	Dichloromethane	16.0±1.2[c]	na	14.0±1.1[c]	33.0±0.4[b,c]
	Ethyl acetate	18.9±1.4[b]	na	11.3±0.7[c,d]	5.8±0.3[d,e]
	n-Butanol	18.1±0.9[b]	14.9±0.8[d]	6.4±0.5[e]	2.5 ±0.3[e]
***Burkholderia* grown on glycerol**	Cyclohexane	na	14.8±1.8[d]	10.7±0.8[d,e]	43.3±1.3[a,b,c]
	Dichloromethane	na	14.7±2.7[d]	9.3±1.2[d,e]	51.9±0.6[a,b]
	Ethyl acetate	na	16.8±2.4[c]	19.7±2.6[b]	62.7±3.2[a]
	n-Butanol	na	23.7±4.2[b]	16.1±2.6[c]	30.2±0.3[b,c]
NDGA (2.0 µg/mL)		53.1±0.1[a]	-	-	-
Galanthamine (1 µg/mL)		-	48.3[a] ±0.1	-	-
Allopurinol (1 µg/mL)		-	-	46.2[a] ±0.2	-
Acarbose (50 µg/mL)		-	-	-	48.4±0.9[a,b]

Data are the means of three independent experiments ± standard deviations (n=3). NDGA: nordihydroguaiaretic acid. na: not active. Means with the same letters in a column are not significantly different at P<0.05.

Anti-α-amylase activity

The evaluation of the anti α-amylase activity of the different extracts of *Burkholderia* sp. was studied for the first time (Table 1).The obtained results showed that all extracts from *Burkholderia* sp. grown on glycerol, exhibited moderate to good anti-α-amylase activity at the concentration of 50 μgml^{-1}. The best percentage of inhibition was observed with the ethyl acetate extract (62.7±3.2%) followed by the dichloromethane one (51.9±0.6%) which both were found to be more active than acarbose (48.4±0.9%) used as a standard. However, we observed that the prepared extracts from *Burkholderia* sp. grown on dextrose exhibited weak to moderate activity with percentage inhibition values ranging from 2.5±0.3 to 33.0±0.4%. The remarkable difference between the activity of the same extract of the strain used but grown on two different sources of carbon (glycerol and dextrose), mainly in the case of ethyl acetate and n-butanol extracts, may be interpreted by the direct effect of the carbon source in the production of specific compounds thus inducing this activity.

Cytotoxic assay

The cytotoxicity of the different extracts at 50µg/mL was assessed for the first time against MCF-7, IGROV, OVACR and HCT-116 cell lines. As shown in Table 2, extracts of *Burkholderia* sp. grown on dextrose exhibited a slight cytotoxic activity against all cell lines used with percentage inhibition values ranging from0 to 40.3±4.8%,but glycerol grown *Burkholderia* sp. were found to be more active (12.6±2.0-63.0±3.2%). The results showed that the cyclohexane extract of *Burrkholderia* sp. grown on glycerol displayed the highest activity against HCT-116 cell line (63.0±3.2%) and was found two time more effective than the same one from *Burkholderia* sp. grown on dextrose (31.6±6.3%). Moreover, the dichloromethane extract (glycerol) exhibited a cytotoxic effect (55.2±4.0%) more than three times compared to the same one from *Burrkholderia* sp. grown on dextrose towards HCT-116. Similarly, the ethyl acetate extract (glycerol) displayed a cytotoxic activity six times more important (52.7±2.7%) than given by the same extract from *Burrkholderia* sp. grown on dextrose already towards HCT-116. It has also been found that MCF-7 cell line was more sensitive towards the cyclohexane extract of *Burrkholderia* sp. (glycerol) than against the same extract of this strain when grown on dextrose which did not display any cytotoxic effect at the used concentration (50 µg/mL). These results allowed to conclude that the production of cytotoxic molecules against the different cell lines used was also affected by the nature of the carbon source added to the culture medium.

Table 2. cytotoxic (MCF-7, IGROV, OVCAR and HCT116 assays) activity of *Burkholderia* sp. extracts. Results are expressed as percentage of enzyme and cell inhibition at 50 mg/L.

Extract		Anti-cancer activity			
		MCF-7	**IGROV**	**OVCAR**	**HCT-116**
***Burkholderia* grown on dextrose**	Cyclohexane	na	28.6 ± 1.1^{c}	17.3 ± 1.8^{b}	$31.6\pm6.3^{c,d}$
	Dichloromethane	$25.2\pm1.6^{c,d}$	14.9 ± 2.2^{d}	na	17.1 ± 2.8^{e}
	Ethyl acetate	$40.3\pm4.8^{b,c}$	22.1 ± 2.8^{d}	18.4 ± 4.3^{b}	8.9 ± 1.3^{f}
	n-Butanol	24.8 ± 9.9^{d}	20.7 ± 2.7^{d}	na	9.6 ± 2.4^{f}
***Burkholderia* grown on glycerol**	Cyclohexane	48.3 ± 1.5^{b}	48.2 ± 0.5^{b}	22.2 ± 2.5^{b}	63.0 ± 3.2^{b}
	Dichloromethane	$40.9\pm0.6^{b,c}$	38.3 ± 1.6^{b}	15.7 ± 0.9^{b}	$55.2\pm4.0^{b,c}$
	Ethyl acetate	$39.8\pm4.9^{b,c}$	34.7 ± 1.3^{b}	17.4 ± 2.4^{b}	52.7 ± 2.7^{c}
	n-Butanol	20.3 ± 4.4^{d}	27.1 ± 3.6^{c}	$12.6\pm2.0^{b,c}$	$32.7\pm1.6^{c,d}$
Tamoxifen (0.2 mg/L)		47.2 ± 4.3^{a}	42.2 ± 1.3^{a}	61.8 ± 4.2^{a}	48.6 ± 2.3^{a}

Data are the means of three independent experiments ± standard deviations (n=3). na: not active. Means with the same letters in a column are not significantly different at $P<0.05$.

Allelopathic potential

Allelochemicals and especially microbial compounds can play an important role as agents that enhance resistance and productivity of plants. This part focused on whether the different organic extracts from a Gram negative bacterium, *Burkholderia* sp., can modulate seed germination. The extracts from the strain grown in two different carbon sources (dextrose and glycerol) were tested against seeds of *Z. mays* (maize) and *H. annuus* (Sunflower). Bioassays in the presence of organic extracts showed that the ethyl acetate and butanolic extracts from *Burkholderia* sp. grown on dextrose were the most effective (Figure 2).

For different seeds, germination index and germination percentage were affected by different extracts. Indeed, the germination percentage varied from 77.1% to 97.9% for both maizeor sunflower seeds. For germination index, values ranged from 30.3 to 40.9 for maize seeds and from 21.1 to 39.5 for sunflower seeds. In presence of the different organic extracts, results showed that corn seeds were more sensitive than sunflower seeds (Figure 4). For germination of corn seeds, results (Figure 3) showed that the n-butanol extract of *Burkholderia* sp. grown on dextrose enhanced significantly maize seeds germination (%G=97.9%), followed by the ethyl acetate extract (glycerol) (%G=91.7%). The results of the percentage of germination of sunflower seeds (Figure3) showed that ethyl acetate and butanol extracts made from dextrose medium enhanced significantly sunflower germination (%G=97.9%), followed by

dichloromethane, ethyl acetate and n-butanol extracts (glycerol)having the same germination of sunflower seeds (%G=95.8%). In contrast, the dichloromethane extract obtained from *Burkholderia* sp. grown on dextrose was the less efficient (%G=77.1%, unlike its behavior with control).

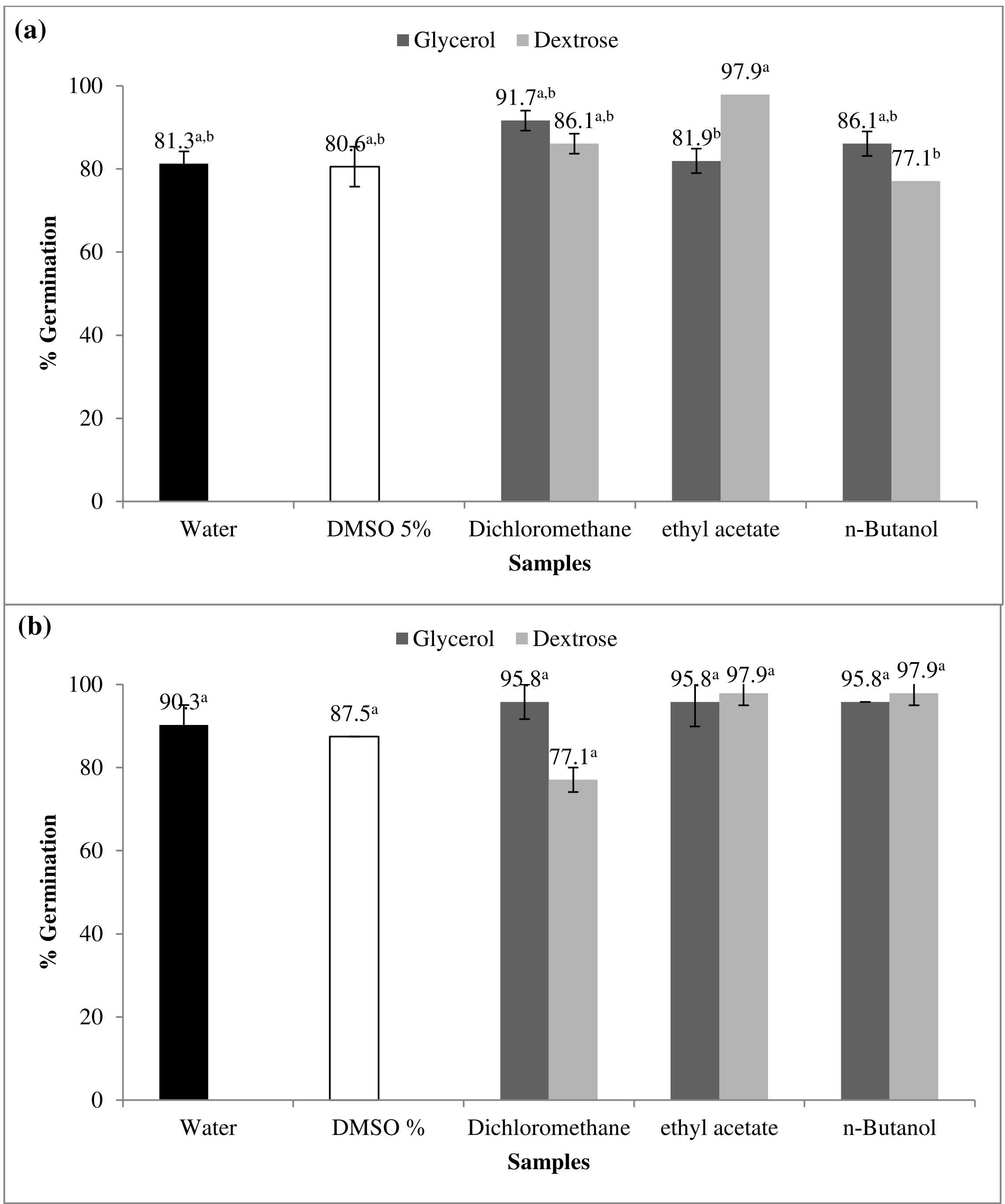

Figure 2. Allelophatic effects of different extracts of *Burkholderia* sp. grown on dextrose and glycerol, expressed as percentage of germination of *Zea mays* (a) and *Helianthus annuus* (b). Means with the same letters are not significantly different at P<0.05.

To our knowledge, no previous studies have been reported on the determination of allelophatic potential of *Burkholderia* sp. extracts for germination of sunflower and corn seeds. So this bacterium specie might be a good raw source of compounds with high potential for plant productivity that could be isolated and developed as agents enhancing plant growth.

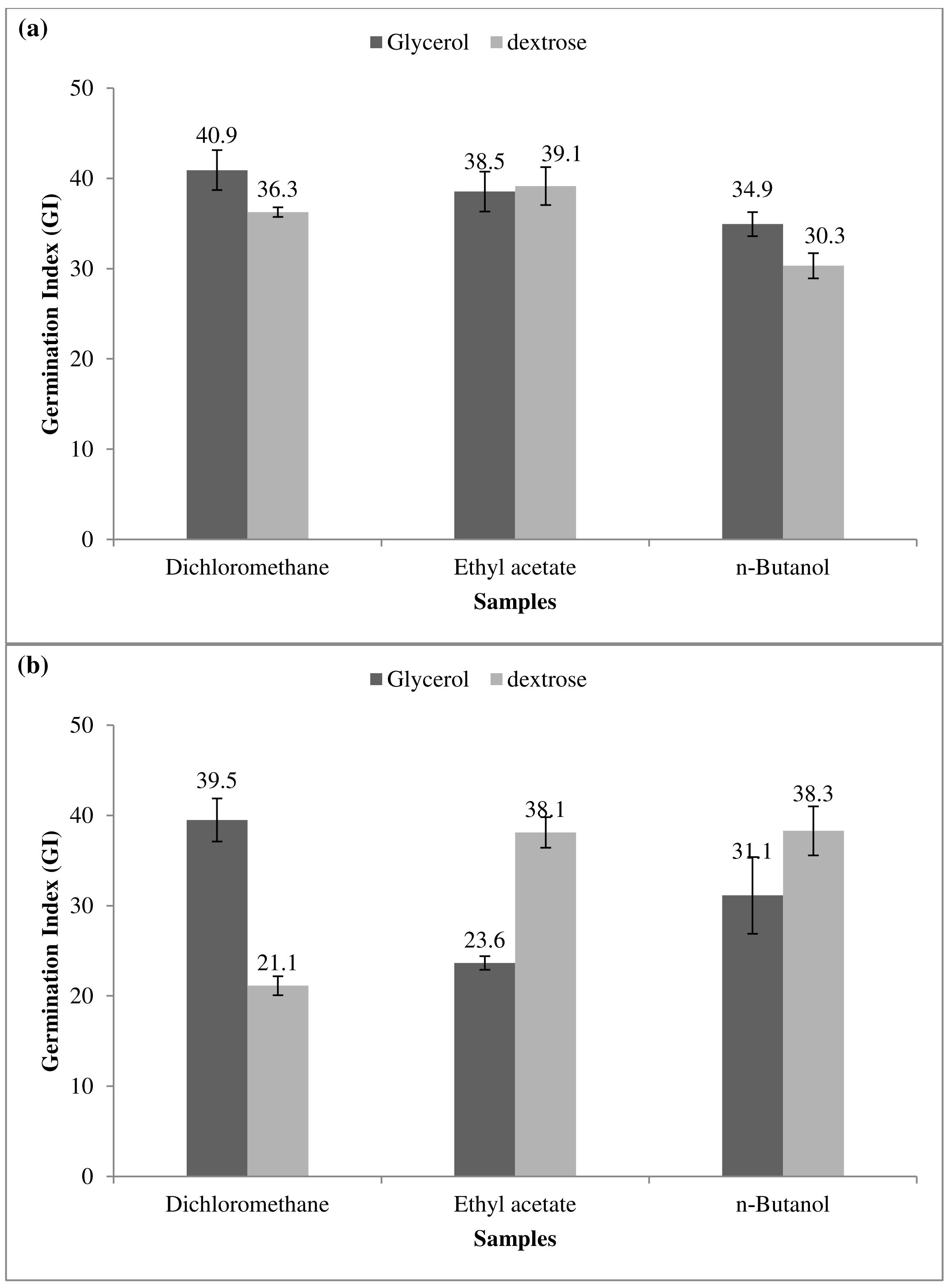

Figure 3. Allelophatic effects of different extracts of *Burkholderia* sp. grown on dextrose and glycerol, expressed asgermination index (GI) of *Zea mays* (a) and *Helianthus annuus* (b).

Conclusion

The present study highlighted the significant influence of the nature of carbon sources in the chemical composition of the secondary metabolites of *Burkholderia* sp. and its significant influence also on the biological activities. The concentrations of phenolic compounds were determined to be highest in *Burkholderia* sp., grown on glycerol, extracts. We can conclude that *Burkholderia* sp. extracts, grown on glycerol, have the highest anti-α-amylase, anti-acetylcholenesterase and cytotoxic activities. However, the highest anti-inflammatory and allelophatic activities were recorded for the *Burkholderia* sp. extracts grown on dextrose. Most of results allowed to show without any doubt the importance of the carbon source added to culture medium of *Burkholderia* sp. to produce specific bioactive molecules. Further studies are in progress to target any specific interesting molecules which may be responsible for the observed biological activities by bio-guided fractionation which open a new horizon about possible utilizations of *Burkholderia* sp. in several fields such as agriculture and pharmaceutical industries.

References

Bekir, J., Mars, M., Vicendo, P., Fterrich, A. and Bouajila, J. (2013) Chemical composition and antioxidant, anti-Inflammatory, and antiproliferation activities of pomegranate (*Punica granatum*) Flowers. *J Med. Food.* 16, 544-550.

Blom, D., Fabbri, C., Connor, E., Schiestl, F., Klauser, D., Boller, T., Eberl, L. and Weisskopf, L. (2011) Production of plant growth modulating volatiles is widespread among rhizosphere bacteria and strongly depends on culture conditions. *Environ. Microbiol.* 13, 3047-3058.

De Soyza A., Silipo, A., Lanzetta, R., Govan, J.R. and Molinaro A. (2008) Chemical and biological features of *Burkholderia cepacia* complex lipopolysaccharides. *Innate Immun.* 14, 127-144.

Elshafie, H.S., Camele, I., Racioppi, R., Scrano, L., Iacobellis, N.S. and Bufo, S.A. (2012) *In vitro* antifungal activity of *Burkholderia gladioli pv. agaricicola* against some phytopathogenic fungi. *Int. J. Mol. Sci.* 13, 16291-16302.

Groenhagen, U., Baumgartner, R., Bailly, A., Gardiner, A., Eberl, L., Schulz, S. and Weisskopf, L. 2013. Production of bioactive volatiles by different *Burkholderia ambifaria* strains. *J. Chem. Ecol.* 39, 892-906.

Hwang, J., Chilton, W.S. and Benson, D.M. (2002) Pyrrolnitrin production by *Burkholderiacepacia*and biocontrol of *Rhizoctonia* stem rot of poinsettia. *Biol. Control.* 25, 56-63.

Kammoun El Euch, S., Bouajila J. and Bouzouita N. (2015) Chemical composition, biological and cytotoxic activities of *Cistus salviifolius* flower buds and leaves extracts. *Ind. Crops Prod.* 76, 1100-1105.

Kanchiswamy C.N., Malnoy M. and Maffei M.E. (2015) Chemical diversity of microbial volatiles and their potential for plant growth and productivity. *Front. Plant Sci.* 6, 151-173.

Omezzine, F., Bouaziz, M., Simmonds, M.S.J. and Haouala R. (2013) Variation in chemical composition and allelopathic potential of mixoploid *Trigonella foenum graecum* L. with developmental stages. *Food Chem.* 148, 188-195.

Shalaby, N.M.M., Abd-Alla, H.I., Aly, H.F., Albalawy, M.A., Shaker, K.H. and Bouajila, J. (2014) Preliminary *in vitro* and *in vivo* evaluation of antidiabetic activity of *Ducrosia anethifolia* Boiss and its linear furanocoumarins. *BioMed. Res. Int.* ID 480545, 1-13.

Vial, L., Marie-Christine G., Valérie, D. and Eric D. (2007) *Burkholderia* diversity and versatility: an inventory of the extracellular products. *J. Microbiol. Biotechn.* 17, 1407-1429.

Wang, J.H., Quan, C.S., Qi, X.H., Li, X. and Fan, S.D. (2010) Determination of diketopiperazines of *Burkholderia cepacia* CF-66 by gas chromatography-mass spectrometry. *Anal. Bioanal. Chem.* 396, 1773-1779.

Conclusion:

Dans ce présent chapitre de thèse, qui entre dans le cadre de recherche des métabolites secondaires microbiens, nous nous sommes intéressés à l'étude de l'influence de la nature de sources de carbone sur la composition chimique (composés phénoliques et composés volatils) de *Burkholderia* sp.AGN02, ainsi que son influence sur les activités biologiques. Pour se faire, nous avons développé plusieurs études dont les résultats obtenus peuvent être résumés comme suit :

- Dans le premier volet de cette partie, nous avons pu ressortir que des extraits de *Burkholderia* sp., cultivé avec du glycérol, contiennent plus de composés phénoliques que ceux de *Burkholderia* sp., cultivé avec du dextrose. Ceci montre bien l'influence notable de la nature de source de carbone sur la production de composés phénoliques.
- Dans le deuxième volet de cette partie, nos résultats indiquent la présence de différentes classes de composés volatils tels que des hydrocarbures, des alcools, des cétones, des esters, des acides carboxyliques et des dicétopipérazines. L'analyse par GC-HRMS nous a permis d'identifier plus que cinquante composés dont *N*-butylbenzenesulfonamide, triacontane et octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate cités pour la premiere fois dans des bactéries.
- Dans le dernier volet, nous avons visé surtout l'influence de la nature de source de carbone sur les activités biologiques des extraits obtenus à partir de *Burkholdria* sp.. Une comparaison des résultats obtenus a révélé que les extraits de *Burkholderia* sp., cultivée avec du glycérol, sont doués des activités anti-α-amylase, anti-acétylcholinestérase et cytotoxiques les plus élevées. Toutefois, l'activité anti-inflammatoire ainsi le potentiel allélopathique les plus élevés ont été enregistrés pour les extraits de *Burkholderia* sp., cultivée avec de dextrose.

Compte tenu de toutes les études chromatographiques, spectroscopiques et biologiques, nous confirmons bien que la nature de source de carbone affecte la production des molécules bioactifs ainsi que les molécules volatiles émises à partir de Burkholderia sp. AGN02.

Chapitre III : Etude de l'effet de l'interaction bactérienne sur la composition de *Burkholderia* sp. et *Bacillus megaterium* et sur les activités biologiques

Introduction

Les microorganismes ont dominé la terre pendant plus de trois milliards d'années et représentent plus de 60% de toute la matière organique sur terre. Ces microorganismes, ubiquités dans notre environnement, ne cessent d'occuper une place de plus en plus importante dans notre vie. Plus récemment, les microorganismes ont servi en tant que des sources remarquables de production de nombreux agents bioactifs et qui sont utilisés pour leurs propriétés fonctionnelles dans les domaines pharmaceutiques ainsi qu'agronomique. Parmi ces agents, on peut citer les antimicrobiens, les antifongiques, les antibiotiques, les anticancéreux, etc.

Dans ce chapitre, notre intérêt s'est porté à l'étude de l'influence des intérractions bacériennes sur la composition chimique de *Burkholderia* sp. et *Bacillus megaterium* et sur les activités biologiques. Ainsi, pour plus de clarté dans la présentation, il est important de noter que les activités ciblées sont : l'activité anti-inflammatoire (anti-5-lipoxygénase), activité anti-xanthine oxydase, activité anti-acétylcholinestérase, l'activité anti-diabétique (anti-α-amylae), la cytotoxicité (MCF-7, HCT-116, OVCAR et IGROVlignées cellulaires à citer) ainsi que le potentiel allélophatique des différents extraits obtenus. Les résultats obtenus ainsi que leurs discussions seront présentées en deux parties comme suit :

- La première partie a été consacrée à l'étude de l'effet des interactions bactériennes sur la production des substances volatiles microbienne à partir de *Burkholderia* sp. et *B. megaterium*. Cette étude a été réalisée en combinant aussi les deux souches bactériennes qui ont montré une capacité de croissance ensemble malgré qu'ils appartiennent à deux Gram différents. Cette partie de thèse fait l'objet d'une publication qui est actuellemnt soumise «*Microbial Ecology*».
- La deuxième partie a été consacrée à l'étude de l'effet des interactions bactériennes sur les activités biologiques de différents extraits obtenus à partir de *Burkholderia* sp., *B. megaterium* et le mélange (combinaison de deux dites bactéries). Cette deuxième partie d'une publication qui est actuellemnt en cours de préparation «*Saudi Journal of Biological Sciences*».

Partie A: Bacterial-bacterial interaction: identification and characterization of microbial volatile organic compounds from *Burkholderia* sp. and *Bacillus megaterium*

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Abstract

Our study treated the influence of external conditions on the production of microbial compounds (MVOCs) by different bacteria. The impact of *Bacillus megaterium* AGN01-*Burkholderia* sp. AGN02 interaction on the production of volatile by each bacterium was presented. Gas chromatography-high resolution mass spectrometry and derivatization reaction were used for the different extracts (cyclohexane, dichloromethane, ethyl acetate and *n*-butanol). More than sixty volatile organic compounds were identified, including diketopiperazines, sulfur containing compounds, furan containing compounds, carboxylic acids and esters including *N*-butylbenzenesulfonamide **9**, triacontane **41**, octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate **42** and (*E*)-5-chloro-3-(hydroxyimino)indolin-2-one **59** reported for the first time from bacteria. In addition, it was found that the combination of the two strains had a negative effect on the emission of volatile compounds; therefore, the secretion of alkaloids, saturated hydrocarbons and esters by *Burkholderia* sp. decreased when the later was combined with *B. megaterium*. Moreover, the skeletons of identified diketopiperrazine and furan containing compounds, known as signaling molecules, were affected by the combination suggesting that these volatile molecules play a key role in bacterial-bacterial interactions. These effects could provide more important clues for elucidating the ecological, chemical and biological significances of microbial volatile compounds emissions and will help to unravel the bacterial-bacterial interactions.

Keywords

Burkholderia sp., *Bacillus megaterium*, MVOCS, chemical composition, Gas chromatography-mass spectrometry, diketopiperazines, derivatization.

Introduction

Microbial volatile organic compounds (MVOCs) are emitted by a wide array of micro-organisms such as fungi and bacteria as a part of their normal metabolism. Recent investigations have demonstrated that MVOCs were produced by bacteria during interactions with other organisms such as plant in order to influence communities (Romoli et al., 2014; Kai et al., 2009). Bacteria are known to affect other organisms and recent studies suggested that MVOCs are an ideal infochemicals because they can act over long distances and over small concentrations which make them playing very important role in bacterial-plant and bacterial-bacterial interactions (Wheatley 2002).

Different strains of *Burkholderia* and *Bacillus* genus were known to secrete a variety of volatile compounds such as hydrocarbons, aromatic compounds, alcohols, ketones, acids and amino containing compounds and many of which have potential therapeutic applications (Kanchiswamy et al., 2015; Groenhagen et al., 2013; Ryu et al., 2004). Many MVOCs have been identified from several *Bacillus* and *Burkholderia* species with different biological properties that make them very useful in industrial practice. For example, pyrrolnitrin; an antibiotic that was first identified in *Burkholderia pyrrocinia* and was later found in other *Burkholderia* species such as *Burkholderia cepacia* strain 55B (Hwang et al., 2002) and it has been used in agriculture fields. *Bacillus* was also able to produce other amino containing compounds such as cyclo(*l*-Pro-*d*-Leu); a diketopiperazine isolated from *Bacillus cereus* having a wide range of biological activities (Strom et al., 2002), in addition to *Bacillus* genera*, Burkholderia* genera was also found to produce diketopiperazine (Jian-Hua et al., 2010). Active volatile molecules of these two bacteria species are generally extracellular, and their identification from the complex fermentation broth needs the use of various separation and analyse steps, such as solvent extraction, gas chromatography, gas chromatography coupled to high performance mass spectrometry and derivatization reaction.

Emission of microbial organic volatiles by different bacteria and fungi species had hardly ever been studied, however and for the first time the impact of the presence of other bacteria on volatile emission was presented in this study. Therefore, the present study describes the identification and characterization of the volatile compounds from different extracts of *Burkholderia* sp. as well as the influence of the volatiles produced by a *B. megaterium* strain and reciprocally by comparing the volatiles emitted by the three different mediums (*Burkholderia sp, B. megaterium* and the mixture of strains).

Material and methods

Identification and culture of studied bacteria

The two studied strains have been isolated from agricultural soil samples used to grow barley and wheat, by the dilution plating technique and their ability to solubilize tricalcium phosphate . These two strains have been characterized morphologically and identified by bacterial 16 S rRNA gene amplification and sequencing by PCR using the universal primer 1492 r and bacterial primer 27f. The resulting sequences were compared by BLAST, search of The National Library of Medicine database (Bethesda, USA) giving an identification as *B. megaterium* AGN01 and *Burkholderia* sp. AGN02. The phylogenetic trees have also been established.

Bacterial strains and culture mediums were obtained from the culture collection of Agronutrition industry (Agronutrition, Labège France). Different microorganisms were grown over dark in a modified bennet broth (water 1000 mL agar 15 g/L, dextrose 10 g/L, peptone 2.5 g/L, pH 7.2) at room temperature. A single colony of each strain from the nutrient agar plate was inoculated into the flask containing 100 ml sterile media. For the mixture, a single colony of *Burkolderia* sp. and *B. megaterium* were mixed and was inoculated into the flask containing 100 ml sterile media. The flasks were then incubated under constant agitation over dark for 24 h to 72h according to the needs of the bacterium. The bacterial mediums were then centrifuged (10,000 ×g, 20 min, 4 °C), the filtered through a 0.22 μm filter, to obtain cell free culture filtrate.

Chemicals used

All chemicals used were of analytical reagent grade. All reagents were purchased from Sigma-Aldrich-Fluka (Saint-Quentin France).

Extraction

The supernatant (5 liters) of each strain was extracted two times using solvents of increasing polarity: cyclohexane (Cyclo), dichloromethane (DCM), ethyl acetate (EtOAc) and *n*-butanol (BuOH). All extracts were evaporated to dryness, using rotary evaporation ((Rotavapor, VWR) at 30°C, to give crudes gummy extracts.

Gas Chromatography-HR Mass Spectrometry

Gas chromatography-high resolution mass spectrometry (GC-HRMS) analyses of each samples were performed with a an HP6890 GC coupled to HRMS (GCT 1er Waters) mass

selective detector fitted with an HP-5 ms fused silica capillary column (30 m, 250 µm i.d. 0.25 µm film) which was piloted by MassLynx.
Helium (99.999%) was used as a carrier gas at a constant flow rate of 1 mL/min. Samples (1 µL) in dichloromethane or methanol were injected into the HRGC–MS. The temperature of the oven was programmed at 70°C and raised at a rate of 10°C/min up to 260°C, followed by 12 minutes isotherm at 260°C, then raised at a rate of 20°C/min up to 300°C and finally held for 16 minutes at 300°C. The high resolution mass had the following conditions: the transfer line from GC to MS was held at 300°C and the trap temperature was 250°C. The detector was operated at 70 eV and the analysis was performed in full scan mode from 30 to 600 *m/z*. The identification of the microbial volatiles compounds were done by determination of their formula obtained from Mass Lynx and by comparison of their resulting *m/z* with those of NIST spectrum library (National Institute of Standards and Technology).

Derivatization method

Using a modified method described by Wenclawiak et al. (1993). Briefly, 150 µL Pierce BSTFA + 1% TMCS was added to extracts (1 mL, 5 mg/mL) in THF anhydrous, and mixed in a 2 mL vial. Then, the mixture was aerated with bubbling using nitrogen and shacked for 30 seconds. The reaction mixture was maintained at 40°C for 15 min. 1 µL of each derivatized solution was finally injected into the GC-MS and analyzed as described in previous section.

Results and discussion

GC-HRMS

To identify the MVOCs produced by all the bacteria used in this study and the effect of bacterial-bacterial interaction on the production of these compounds, the gas chromatography coupled with high resolution mass spectrometry was used. The analysis was performed on *Burkholderia* sp., *B. megaterium* and the mixture of the two bacteria to check whether the MVOCs production by one strain might be induced and/or modified by the presence of the other one. Results obtained are reported in Table 1 and revealed that MVOCs profiles from the three culture mediums were very different. Moreover, the volatile profiles from the different culture showed the presence of a large variety of organic compound classes such as saturated and unsaturated hydrocarbons, alcohols, carboxylic acids, esters, aromatic compounds, sulfur compounds, amino compounds and cyclic peptides (Table 1, Figures 1 and 2). A list of fourty-two compounds was identified from the different samples. It can be

noticed that two of these compounds are sulfur-containing ones and were detected only on the cyclohexane extract from the *Burkholerdia* sp. strain and were identified as *N*-butylbenzenesulfonamide **9** and 4,4'-thiobis(2-(*tert*-butyl)-5-methylphenol **38**. These sulfur compounds are probably the responsible for the characteristic smell of this strain (Groenhagen et al., 2013). Beside sulfur compounds, a long chain saturated hydrocarbons such as tricosane **27** and triacontane **41** were only detected on *Burkholderia* sp. cyclohexane extract. Schulz and Dickschat (2007) and Uta et al.. (2012) indicated that bacteria belonging to *Pseudomonas* species were able to produce saturated and unsaturated hydrocarbons. In the other side, neither *B. megaterium* nor the mixture were able to produce saturated hydrocarbons, however one unsaturated hydrocarbon was identified as eicosene **20** was identified in *B. megaterium* ethyl acetate extract. Furthermore, a plethora of esters was produced by the strains. Several esters as methyl palmitate **15**, methyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate **16**, methyl 14-methylheptadecanoate **23** and dodecyl octanoate **24**, were identified only from *Burkholdria* sp. cyclohexane extract. In contrast, (*E*)-methyl octadec-9-enoate **22** was found in the *Burkholderia* sp. cyclohexane extract and in ethyl acetate from the mixture. Octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate **42** was common for both cyclohexane extract from *Burkholderia* sp. and *B. megaterium*. Also, an oxaspiro lactone was emitted only by the *Burkholderia* sp. strain (Cyclo) and identified as 7,9-di-*tert*-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione **13**. These results showed that the number of esters detected in the mixture decreased, so we can conclude that the production of esters by the *Burkholderia* sp. strain has been affected by the presence of the *B. megaterium* which reduce the production of this class of molecules by *Burkholderia* sp.. Several linear alkaloids compounds like *N*,*N*-dimethyl-tetradecan-1-amine **1** and *N*,*N*-dimethyldodecan-1-amine **5** produced only by the *Burkholderia* sp. strain, were identified in the cyclohexane extract. Several studies indicate that amino compounds, produced by bacteria, can promote plant growth (Velazquez-Becerra et al., 2011). Another group of alkaloids was the diketopiperazine, which is the smallest cyclic peptides known and which represents one of major group of organic volatiles emitted by bacteria (Schulz and Dickschat 2007). As other bacteria, more than one diketopiperazine (Figure 1) were produced by the strains studied here. Moreover, some diketopiperazines (same mass spectra) were eluted with different retention times and attributed to *Dextrogyre* and *Levogyre* isomers (Jian-Hua et al., 2010). 3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (**28**, **31** and **33**) (DCM) were produced by the two strains and the mixture. However, the 3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (**14** and **17**) (cyclo, DCM and EtOAc) were detected only in the *Burkholderia* sp. and *B. megaterium* strains. 3-

methylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione **6** (DCM) and 3-propylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (**10** and **11**) (DCM and EtOAc) were produced by only the *Burkholderia* sp. strain. Three other diketopiperazines identified as the 3-benzyl-6-methyl-2,5-piperazinedione **25**, 3-benzyl-6-isopropyl-2,5-piperazinedione **26** and 3-benzyl-6-isobutyl-2,5-piperazinedione **29** were emitted by only the *B. megaterium* strain (DCM). According to previous studies (Skwierczynski et al., 1993; Jian-Hua et al., 2010), this class of compounds can be generated by the complex medium used to culture our bacteria including yeasts. A negative control test allowed us to detect effectively a small amount of 3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (**14** and **17**) and 3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione **31**. This evidence that 3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (**14** and **17**) and 3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione **31** not only produced by our bacteria but also by the medium. However, diketopiperazines formed by bacteria are much higher than the diketopiperazines produced by the medium. In conclusion, seven diketopiperzaines were identified in our strains used in this study, and result showed that the secretion of this class of compounds by the studied bacteria has been affected by the presence of other bacteria. In 2004, Barnard and Salmon have classified this class of molecules as signal molecules, so we can deduced that the secretion of these cyclic peptides was related to the state of the bacteria (single or in presence of other bacteria) however the role of their secretion still largely unknown in signal transduction.

hexahydropyrrolo[1,2-a]pyrazine-1,4-dione[(a)] 3-methylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione[(a)] 3-propylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione[(a)]

3-benzylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione[(a,b)]

3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione[(a,b)]

3-Benzyl-6-isobutyl-2,5-piperazinedione[(b)] 3-Benzyl-6-methyl-2,5-piperazinedione[(b)] 3-Benzyl-6-isopropyl-2,5-piperazinedione[(b)]

Figure 1. Different diketopiperazine (DKP) emitted by *Burkholderia* sp. AGN02 (a) and *Bacillus megaterium* AGN01 (b).

Despite the decrease of the number of detected diketopiperazines in the mixture mediums, we notice that the later contain the higher quantities of diketopiperazine derivatives (area or hiehgt mV). The emitting of this class of compounds, known as signal molecules, can be an indicator of emitting different response to *Burkholderia*-*B. megaterium* interaction.

In conclusion, a list of fourty-two compounds were identified from the different extracts of the studied strains; therefore, prior to this study, there were no reports on the literature on the production of *N*-butylbenzenesulfonamide **9**, 7,9-di-*tert*-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione **13**, methyl palmitate **15**, dodecyl octanoate **24**, 4,4'-thiobis(2-(*tert*-butyl)-5-methylphenol) **38**, octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate **42** and all saturated hydrocarbons (**27**, **32**, **34**, **36**, **37**, **39**, **40** and **41**) by bacteria. In addition, there were no report recorded the production of *N,N*-dimethyldodecan-1-amine **1**, *N,N*-dimethyltetradecan-1-amine **5**, 3-methylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione **6**, 3-propylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (**10** and **11**), hexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione **18**, *N*-benzyl-2-phenylacetamide **21**, (*E*)-methyl octadec-9-enoate **22** and methyl 14-methylheptadecanoate **23** by bacteria of the species *Burkholderia*. All the rest of identified compounds were reported for the first time in *B. megaterium* and *Burkholderia* sp. but reported from other *Bacillus* and/or *Burkholderia* species (Bitas et al., 2013; Jian-Hua et al., 2010; Kanchiswamy et al., 2015; Leyton et al., 2012; Uta et al., 2012).

Table 1. GC-HRMS analysis of different extracts of *Burkholderia* sp., *Bacillus megaterium* and combined strains.

N°	rt (min)	Compound	*Burkholderia* sp.				*Bacillus megaterium*				Mixture			
			Cyclo	DCM	EtOAc	BuOH	Cyclo	DCM	EtOAc	BuOH	Cyclo	DCM	EtOAc	BuOH
1	10.96	*N,N*-dimethyldodecan-1-amine	×											
2	11.01	2,4-di-*tert*-butylphenol	×						×					
3	11.01	2,4-di-*tert*-butylphenyl 5-hydroxypentanoate	×						×					
4	11.87	1-hexadecanol							×					
5	13.31	*N,N*-dimethyltetradecan-1-amine	×											

6	13.47	3-methylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione		×			
7	13.64	(2*S*)-2-(2-aminopropanamido)-4-methylpentanoic acid			×		
8	14.13	1-octadecanol				×	
9	14.25	*N*-butylbenzenesulfonamide	×				
10	14.59	3-propylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-		×	×		×

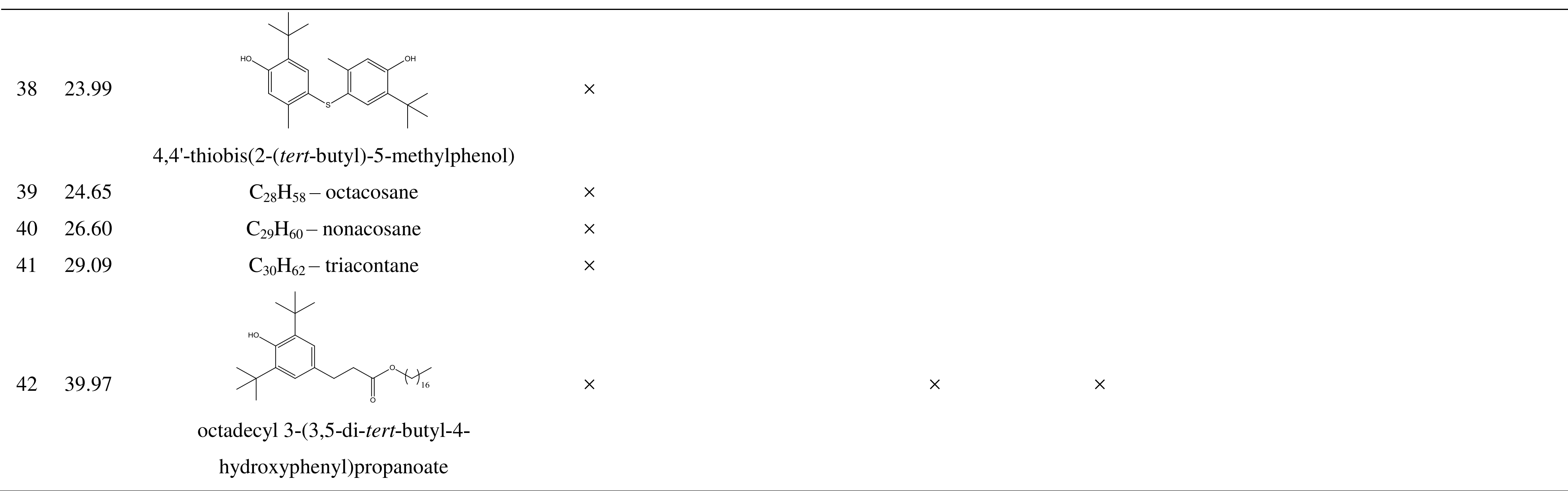
		dione(dextro or levo)				
11	14.87	3-propylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione(dextro or levo)		×		
12	15.00	diisobutyl phthalate	×			
13	15.47	7,9-di-*tert*-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	×			
14	15.56		×	×	×	×

		3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione* (dextro or levo)					
15	15.64	methyl palmitate	×				
16	15.70	methyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate	×				
17	15.76	3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione* (dextro or levo)		×	×	×	×
18	15.84	hexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione			×		

19	15.97	dibutyl phthalate	×					
20	16.19	eicosene				×		
21	16.45	*N*-benzyl-2-phenylacetamide	×					
22	17.39	(*E*)-methyl octadec-9-enoate	×					×
23	17.57	methyl 14-methylheptadecanoate	×					
24	18.01	dodecyl octanoate	×					

25	18.03	3-benzyl-6-methyl-2,5-piperazinedione		×	
26	18.98	3-Benzyl-6-isopropyl-2,5-piperazinedione		×	
27	19.11	$C_{23}H_{48}$ – Tricosane	×		
28	19.55	3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (dextro or levo)		×	×
29	19.57	3-benzyl-6-isobutyl-2,5-piperazinedione (dextro or levo)		×	
30	19.71	3-benzyl-6-isobutyl-2,5-piperazinedione (dextro or levo)		×	

31	19.86	3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione* (dextro or levo)		×								×
32	19.94	$C_{24}H_{50}$ – tetracosane	×									
33	19.95	3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione(dextro or levo)						×				
34	20.82	$C_{25}H_{52}$ – pentacosane	×									
35	21.12	bis(2-ethylhexyl) phthalate	×									
36	21.85	$C_{26}H_{54}$ – hexacosane	×									
37	23.10	$C_{27}H_{56}$ – heptacosane	×									



38	23.99	4,4'-thiobis(2-(*tert*-butyl)-5-methylphenol)	×					
39	24.65	$C_{28}H_{58}$ – octacosane	×					
40	26.60	$C_{29}H_{60}$ – nonacosane	×					
41	29.09	$C_{30}H_{62}$ – triacontane	×					
42	39.97	octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate	×		×	×		

*: present in non-inoculated culture media.

GC/HRMS: chemical derivatization

To identify other compounds, derivatization reaction was applied for different extracts. This step had led to the identification of twenty-six compounds (Table 2) including (*E*)-5-chloro-3-(hydroxyimino)indolin-2-one **59** reported for the first time from bacteria, 5-(1,2-dihydroxyethyl)-4-hydroxydihydrofuran-2(3*H*)-one **66** and 1-(3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1*H*,3*H*)-dione **68** reported for the first time from a *Burkholderia* species and all the rest of identified silylated compounds were reported for the first time in *Bacillus megaterium* and *Burkholderia* sp. but reported from other *Bacillus* and *Burkholderia* species (Bitas et al., 2013; Gutierrez-Luna et al., 2010; Kanchiswamy et al., 2015; Rudrapa et al., 2010; Uta et al., 2012).

Results showed that a plethora of alcohols was produced by all strains. Several alcohols like, glycerol **57** (DCM, EtOAc and BuOH) and butan-1,2,3-triol **56** (DCM, EtOAc and BuOH) were common for all strains. Butan-2,3-diol **50** (DCM, EtOAc and BuOH) known also as 2,3-BD and pentan-2-ol **51** (DCM and EtOAc) were detected only on the *Burkholderia* sp. and *B. megaterium* strains, however, a isomer of butan-2,3-diol which was identified as butan-1,3-diol **55**, was detected only in the mixture ethyl acetate extract. Other alcohols such as propan-1,2-diol **49** (EtOAc) and ethan-1,2-diol **48** (DCM) were only emitted by *Burkholderia* sp. 3-Methylbutan-2-ol **53** and 3-methylbutan-1,2-diol **58** were also identified but only in the *B. megaterium* (EtOAc). Alcohols were known for their interference with plant and stimulation of plant growth (Kai et al., 2010; Blom et al., 2011). On the other hand, carboxylic acids (Figure 3) have been also identified such as, palmitic acid **67**, 3,4,5-trihydroxypentanoic acid **61** and (*R*)-3-hydroxybutanoic acid **54** which were emitted only by *Burkholderia* sp. strain (DCM and BuOH), however, the last two carboxylic acids were also detected in the supernatant of the mixture (DCM, EtOAc and BuOH); so it is certain that these acids were produced by *Burkholderia* sp. Two other acids 2-methylbutanoic **45** and 3-methylbutanoic **46** were detected both in *Burkholderia* and *Bacillus* strains (DCM and EtOAc). Carboxylic acids are known to be produced by microorganisms to be also used in the control postharvest plant diseases (Mercier and Jimenez 2004; Kanchiswamy et al., 2015). In addition to alcohols and carboxylic acids, pyrimidine compounds occurred as well: pyrimidin-2,4-diol **60** and 3-methylpyridin-2,6-diol **62** in *B. megaterium* medium culture (EtOAc). The 3-hydroxybutan-2-one (**43** and **52**) (DCM) also known as acetoin was found in the two strains separately, in contrast to 5-(1,2-dihydroxyethyl)-4-hydroxydihydrofuran-2(3*H*)-one **66** and 1-(3,4-dihydroxy-5-(hydroxymethyl)-tetrahydrofuran-2-yl)pyrimidine-2,4(1*H*,3*H*)-dione **68**, two furan containing compounds detected in *Burkholderia* sp. butanolic extract. A derived

acetophenone was detected in *B. megaterium* ethyl acetate extract and was identified as 1-(2,6-dihydroxyphenyl)ethanone **47**.

By direct or indirect analysis by GC-HRMS, we identified components from huge variety of chemical classes in every strain, although, a great differences in the volatile profiles, obtained from the two strains tested separately and from combined strains, were observed which confirm the presence of a correlation between bacterial-bacterial interaction and the emission of microbial volatile organic compounds. It is not surprising to find different blends of volatiles produced by the two strains, tested separately, given that the two tested strains belong to different Gram bacteria. Moreover, after combination of the two bacteria, we showed that a great influence of *Burkholderia-Bacillus* interaction on the production of volatiles was observed. The most interesting substance classes are the diketopiperazines furan containing compounds, known as signal molecules, esters and alcohols. All the volatile diketopiperazines produced by *Burkholderia* sp. contained a proline moiety such as 3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (**14** and **17**) and 3-methyllhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione **6**. However almost all diketopierazines, identified in the mixture and in *B. megaterium* strains, contained a phenylalanine moiety, except 3-propylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (**10** and **11**) and 3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (**14** and **17**) which didn't contain a phenylalanine moiety. This can be an indicator of emitting different response, especially that this class of compounds was classified as signal molecules (Wang et al., 2010). In addition to diketopiperazine, furan containing compounds, another class of molecule able to interfere with bacteria (Kanchiswami et al., 2015), was identified but only in *Burkholderia* sp. and the mixture. The structures of these compounds were different which can be also another indicator of emitting different response to bacterial interaction, especially that this class of compounds was also classified as signal molecules interfere with bacteria (Zou et al., 2010; Kanchiswami et al., 2015).

The highly represented classes of compounds detected were esters, including aliphatic and aromatic esters. *Burkholderia* sp. was able to produce both aliphatic and aromatic esters such as methyl palmitate **15** and methyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate **16**. However, only one aromatic ester (octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate **42**) was identified in *B. megaterium* strain, in contrast, the mixture was able to produce only one aliphatic ester identified as (*E*)-methyl octadec-9-enoate **22**. This also can be an indicator of emitting different response due to the bacterial-bacterial interaction, especially that this class of compounds was known for its ability to induce

bacterial responses (Kanchiswami et al., 2015; Smaoui et al., 2011). All results showed that the combination of *B. megaterium* and *Burkholderia* sp., influences the secretion of volatile molecules; therefore, the secretion of alkaloids, saturated hydrocarbons and esters by *Burkholderia* sp. decreased when the later was combined with *B. megaterium*. In addition, the skeleton of detected diketopiperazines, esters and furan containing compounds, known for their ability to induce bacterial responses, was different which makes us suggesting that MVOCs play a key role in bacterial-bacterial interactions.

2-methylbutanoic acid [a,b]

3-methylbutanoic acid [a,b]

(*R*)-3-hydroxybutanoic acid [a,b]

3,4,5-trihydroxypentanoic acid [a,c]

propan-1,2-diol [a]

3-methylbutan-1,2-diol [c]

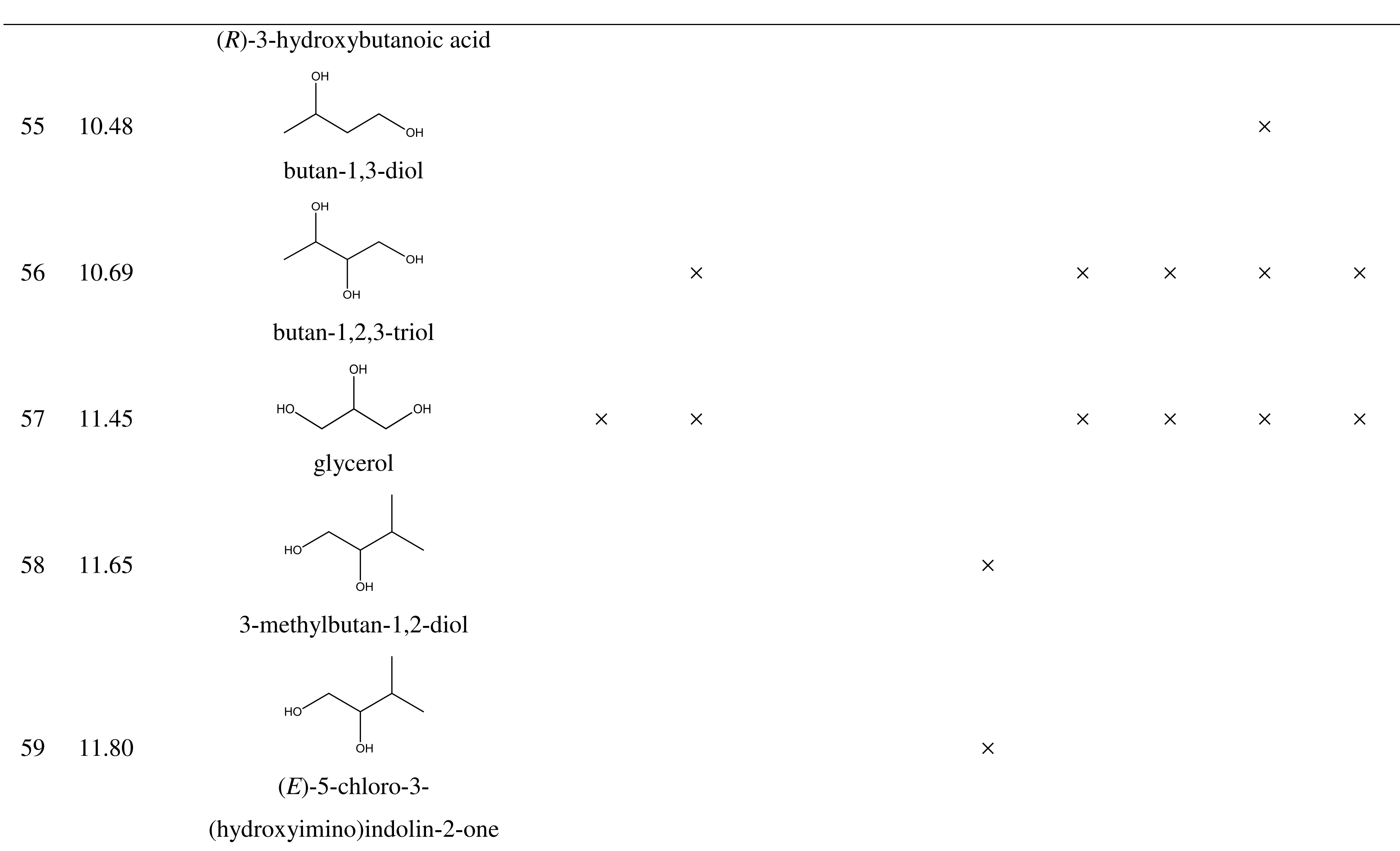
palmitic acid [a]

Figure 2. Carboxylic acids and alcohols produced by *Burkholderia* sp. AGN02 (a), *Bacillus megaterium* AGN01 (b) and their mixture (c).

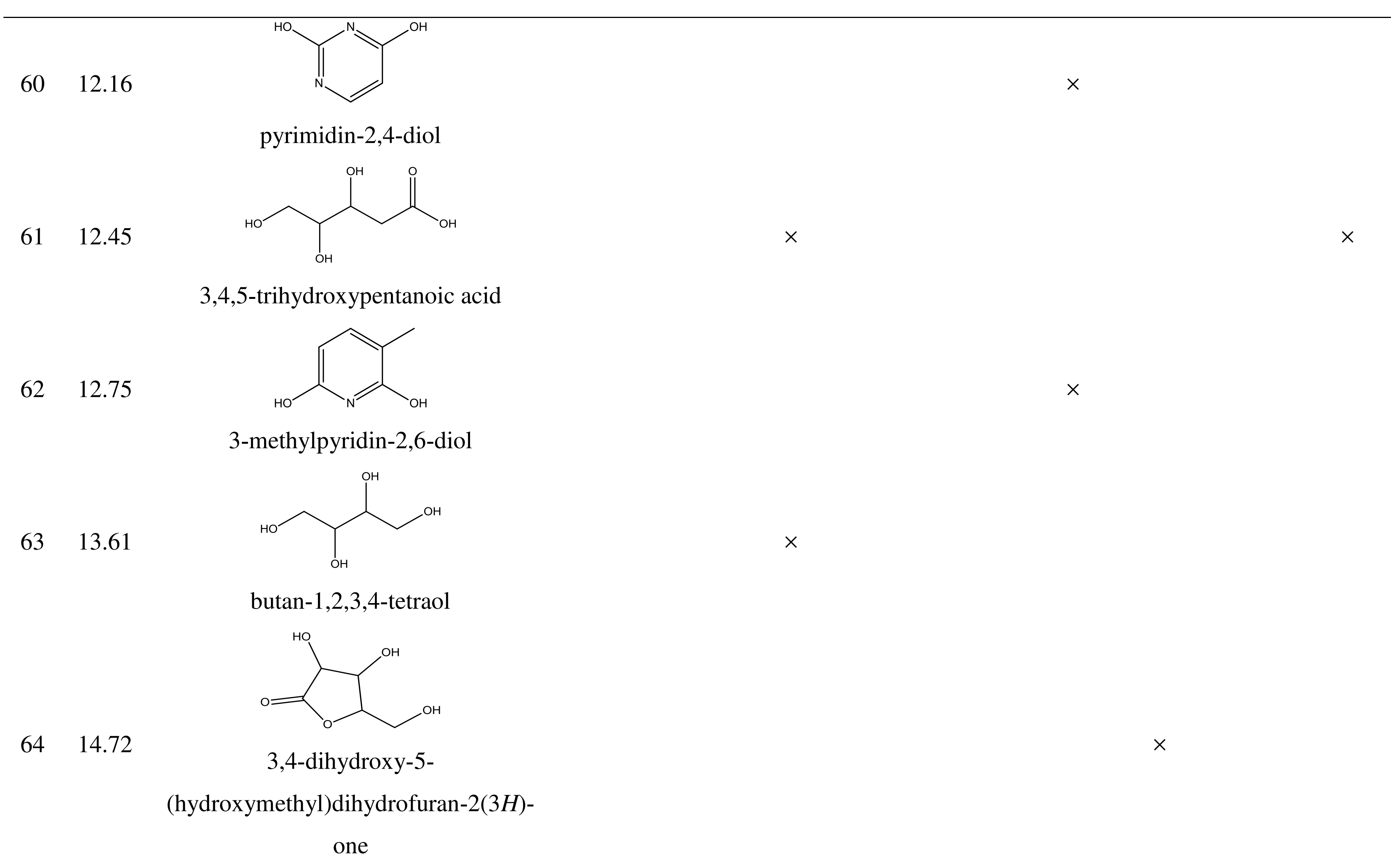
Table 2. Compounds identified in different extracts of *Burkholderia* sp., *Bacillus megaterium* and combined strains after silylation.

N°	rt (min)	Compound	*Burkholderia* sp.			*Bacillus megaterium*			Mixture		
			DCM	EtOAc	BuOH	DCM	EtOAc	BuOH	DCM	EtOAc	BuOH
43	5.71	3-hydroxybutan-2-one (*R* or *S*)	×			×	×				
44	6.22	3-hydroxybutan-2-yl acetate		×		×		×			
45	6.47	2-methylbutanoic acid	×	×			×				
46	6.71	3-methylbutanoic acid	×	×		×					
47	7.75	1-(2,6-dihydroxyphenyl)ethanone					×				

48	7.82	ethan-1,2-diol			×					
49	7.99	propan-1,2-diol		×						
50	8.53	butan-2,3-diol	×	×	×	×	×	×		
51	9.43	pentan-2-ol	×				×			
52	9.65	3-hydroxybutan-2-one (*R* or *S*)	×							
53	9.70	3-methylbutan-2-ol					×			
54	10.22		×		×				×	×



		(*R*)-3-hydroxybutanoic acid									
55	10.48	butan-1,3-diol								×	
56	10.69	butan-1,2,3-triol		×				×	×	×	×
57	11.45	glycerol	×	×				×	×	×	×
58	11.65	3-methylbutan-1,2-diol					×				
59	11.80	(*E*)-5-chloro-3-(hydroxyimino)indolin-2-one					×				



60	12.16	pyrimidin-2,4-diol		×		
61	12.45	3,4,5-trihydroxypentanoic acid	×			×
62	12.75	3-methylpyridin-2,6-diol		×		
63	13.61	butan-1,2,3,4-tetraol	×			
64	14.72	3,4-dihydroxy-5-(hydroxymethyl)dihydrofuran-2(3*H*)-one			×	

65	16.19	*N*-(3,4,5,6-tetrahydroxy-1-oxohexan-2-yl)acetamide			×
66	17.33	5-(1,2-dihydroxyethyl)-4-hydroxydihydrofuran-2(3*H*)-one		×	
67	17.52	palmitic acid	×		
68	20.34	1-(3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1*H*,3*H*)-dione		×	

Conclusion

The aim of our study was to determine whether volatile emission of a *Burkholderia* strain would be influenced by the presence of *B. megaterium* strain and reciprocally. Studied strains and their mixture were investigated for their chemical composition by the determination of their volatile profiles in different extracts prepared (cyclohexane, dichloromethane, ethyl acetate and *n*-butanol) using GC-HRMS and derivatization reaction. It was found that more than sixty volatile organic compounds were identified from the different extracts and different classes of chemical compounds were present such as sulfur containing compounds, furan containing compounds, ketones, diketopiperazines and carboxylic acids. The obtained results stated that the combination of *Burkholderia* and *B. megaterium* influenced the production of blends of volatiles emitted by the two strains. Further studies are necessary for quantifying detected compounds and testing their bioactivity which may be promising candidates for agriculture and pharmaceutical applications.

References

Barnard, A. M. L., and Salmond, G. P. C. 2004. Quorum sensing: the complexities of chemical communication between bacteria. *Complexus*. 5: 87-101.

Bitas, V., Kim, H.S., Bennet, J.W., Kang, S. 2013. Sniffing on Microbes: Diverse Roles of Microbial Volatile Organic Compounds in Plant Health. *Mol Plant Microbe In*. 26, 835-843.

Blom, D., Fabbri, C., Connor, E., Schiestl, F., Klauser, D., Boller, T., Eberl, L., and Weisskopf, L. 2011. Production of plant growth modulating volatiles is widespread among rhizosphere bacteria and strongly depends on culture conditions. *Environ. Microbiol.* 13, 3047-3058.

Garbeva, P., Hordijk, C., Gerards, S., De Boer, W. 2014. Volatile-mediated interactions between phylogenetically different soil bacteria. *Front. Microbiol.* 5, 289.

Groenhagen U., Baumgartner R., Bailly A., Gardiner A., Eberl L., Schulz S., Weisskopf L. 2013. Production of bioactive volatiles by different *Burkholderia ambifaria* strains. *J. Chem. Ecol.* 39, 892-906.

Gutierrez-Luna, F. M., Lopez-Bucio, J., tamirano-Hernandez, J., Valencia-Cantero, E., de la Cruz, H.R., Ias-Rodriguez, L. 2010. Plant growth-promoting rhizobacteria modulate root system architecture in *Arabidopsis thaliana* through volatile organic compound emission. *Symbiosis*. 51, 75-83.

Hwang, J., Chilton, W. S., Benson, D. M. 2002. Pyrrolnitrin production by *Burkholderia cepacia* and biocontrol of *Rhizoctonia* stem rot of poinsettia. *Biol.Control*. 25, 56-63.

Jian-Hua, W., Chun-Shan, Q., Xiao-Hui, Q., Xin, L., Sheng-Di, F., 2010. Determination of diketopiperazines of *Burkholderia cepacia* CF-66 by gas chromatography–mass spectrometry. *Anal. Bioanal. Chem.* 396: 1773–1779.

Kai, M., Crespo, E., Cristescu, S.M., Harren, F.J., Francke, W., and Piechulla, B. 2010. *Serratia odorifera*: analysis of volatile emission and biological impact of volatile compounds on *Arabidopsis thaliana*. *Appl. Microbiol. Biotechnol.* 88: 965-976.

Kai M., Haustein M., Molina F., Petri A., Scholz B., Piechulla B., 2009. Bacterial volatiles and their action potential. *Appl. Microbiol. Biotechnol.* 81, 1001-1012.

Kammoun El Euch, S., Bouajila J., Bouzouita N., 2015. Chemical composition, biological and cytotoxic activities of *Cistus salviifolius* flower buds and leaves extracts. *Ind. Crops Prod.* 76: 1100–1105.

Kanchiswami, C., N., Malnoy, M., Maffei, M. E. 2015. Bioprospecting bacterial and fungal volatiles for sustainable agriculture. *Trends Plant Sci.* In press DOI:10.1016/ j.tplants.2015.01.004

Kanchiswamy C. N., Malnoy M., Maffei M. E., 2015. Chemical diversity of microbial volatiles and their potential for plant growth and productivity. *Front. Plant Sci.* 6, 151.

Leyton, Y., Borquez, J., Darias, J., Cueto, M., Ana, R.D.M., Riquelme, C. 2012. Diketopiperazines produced by a *Bacillus* species inhibits *Vibrio Parahaemolyticus*. *Aquac Res Dev*. 3, 144-148..Mercier, J. and Jimenez, J. I. 2004. Control of fungal decay of apples and peaches by the biofumigant fungus *Muscodor albus*. *Postharv. Biol. Technol.* 31, 1-8.

Ren, Q., Ruth, K., Thoeny-Meyer, L., Zinn, M. 2010. Enatiomerically pure hydroxycarboxylic acids: current approaches and future perspectives. Appl MicrobiolBiotechnol 87(1):41–52.

Romoli R., Papaleo M., De Pascale D., Tutino M., Michaud L., Lo Giudice A., Fani R., Bartolucci G., 2014. GC-MS volatolomic approach to study the antimicrobial activity of the antarctic bacterium *Pseudoalteromonas sp* TB41. *Metabolomics*. 10, 42-51.

Rudrappa, T., Biedrzycki, M. L., Kunjeti, S. G., Donofrio, N. M., Czymmek, K. J., Pare, P. W. 2010. The rhizobacterial elicitor acetoin induces systemic resistance in *Arabidopsis thaliana*. *Commun. Integr. Biol.* 3: 130-138.

Ryu C. M., Farag M. A., Hu C. H., Reddy M. S., Kloepper J. W., Pare P. W., 2004. Bacterial volatiles induce systemic resistance in Arabidopsis. *Plant Physiol.* 134: 1017-1026.

Schulz, S. and Dickschat, J. S. 2007. Bacterial volatiles: the smell of small organisms. *Nat. Prod. Rep.* 24: 814-842.

Skwierczynski, R. D. and Connors, K. A. 1993. Demethylation kinetics of aspartame and L-phenylalanine methyl-ester in aqueous solution. *Pharm Res.* 10: 1174-1180.

Smaoui ,S., Mellouli, L., Lebrihi, A., Coppel, Y., Fguira, L.F., Mathieu, F. 2011. Purification and structure elucidation of three naturally bioactive molecules from the new terrestrial *Streptomyces* sp. TN17 strain. *Nat Prod Res*. 25(8): 806-814.

Strom, K., Sjogren, J., Broberg, A. Schnurer, J. 2002. *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(*L*-Phe-*L*-Pro) and cyclo(*L*-Phe-trans-4-OH-*L*-Pro) and 3-phenyllactic acid. *Appl. Environ. Microbiol.* 68: 4322–4327.

Uta, E.T, Janine, K., Rene, W., Birgit, P. 2012. Volatile mediated interactions between bacteria and fungi in the soil. *J. Chem. Ecol*. 38:665-703.

Velazquez-Becerra, C., Iveth, M. R. L., Lopez-Bucio, J., tamirano-Hernandez, J., Flores-Cortez, I., Valencia-Cantero, E. 2011. A volatile organic compound analysis from *Arthrobacter agilis* identifies dimethylhexadecylamine, an amino-containing lipid modulating bacterial growth and *Medicago sativa* morphogenesis in vitro. *Plant and Soil*. 339, 329-340.

Wheatley, R. E., 2002. The consequences of volatile organic compound mediated bacterial and fungal interactions. *Antonie Van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* 81, 357-364.

Wang, Y., Liu, S. 2014. Production of (*R*)-3-hydroxybutyric acid by *Burkholderia cepacia* from wood extract hydrolysates. *AMB Express*. 4:28.

Wang, J., H., Quan, C., S., Qi, X., H., Li, X., Fan, S., D. 2010. Determination of diketopiperazines of *Burkholderia cepacia* CF-66 by gas chromatography-mass spectrometry. *Analytical and Bioanalytical Chemistry* 396, 1773-1779.

Wenclawiak, B., W., Jensen, T., E., Richert, J., F., O. 1993. GC-MS-FID Analysis of BSTFA Derivatized Polar Components of Diesel Particulate Matter (NBS SRM 1650) Extract. *Fresen. J. Anal. Chem.*. 6, 808-812.

Zou, C., Li, Z., Yu, D. 2010. *Bacillus megaterium* strain XTBG34 promotes plant growth by producing 2-Pentylfuran. *J. Microbiol.* 48, 460-466.

Partie B: Influence of bacterial-bacterial interaction on the chemical composition, allelochemical effects and biological activities of *Burkholderia* and *Bacillus megaterium* strains

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Abstract

Bacteria were generally considered purely selfish organisms and interactions between those inhabiting soils were antagonistic, however, some species can act synergistically and can share task. In addition, a new *Burkholderia* specie and *Bacillus megaterium* strain were able to inhabit the same niche. The influence of the interaction of these two bacteria on the chemical composition (total phenolic content, high performance liquid chromatography analysis (HPLC)), allelophatic potential, anti-inflammatory (anti-5-lipoxygenase), anti-xanthine oxidase (XOD), anti-diabetic (anti-α-amylase), anti-acetylcholinesterase (AChE), and cytotoxic (IGROV, OVCAR, HCT-116 and MCF-7) activities was investigated. HPLC with UV detection was employed for the determination of aromatic and phenolic compounds. The UV chromatograph showed a great difference in the composition of different extracts with a negative impact of *Burkhholderia* sp.-*Bacillus megaterium* interaction on the production of these compounds. Moreover, results showed that the highest phenolics content (28.7 g GAE/kg of dry mass) and strongest anti-xanthine oxidase (IP%=48.8%) were obtained for *B. megaterium* *n*-butanol and dichloromethane extracts respectively. Moreover cyclohexane extract from the same strain possessed more evident cytotoxic activity against MCF-7, IGROV, OVCAR and HCT-116 cell lines (IP%=98.3, 96.5, 60.0 and 99.2%, respectively). It was found that a clear increase in the anti-diabetic and anti-inflammatory activities was observed for the mixture of the two strains; the strongest activities were obtained by the dichloromethane extract (IP%=73.5%) and the *n*-butanol extract (IP%=36.8%) respectively. However, the highest potential for plant growth was obtained in *Burkholderia* sp. ethyl acetate extract (% Germination=97.92% towards maize and sunflower seeds). Results showed that the interaction between bacteria influenced considerably the chemical composition content, the agronomic and the biological activities of *Burkholderia* sp. and *B. megaterium.*

Keywords

Burkholderia sp., *Bacillus megaterium*, synergetic, antagonistic, HPLC, anti-diabetic, anti-cancer.

Introduction

For more than a century, bacteria were leading producers of useful compounds that have cured and/or reduced the effect of not only plant diseases but also human diseases. Bacterial products including antibiotics, antivirals, antitumors, insecticides and plant growth regulators were used in the treatments of large variety of diseases and through it helped to revolution the practice of pharmacy, medicine and agriculture.

Among bacteria, *B. megaterium* a Gram positive bacterium belongs to the larger bacteria with its eponymous size (De Bary, 1884) is widely used in industrial field as a producer of various amylases and penicillin acylases (Takasaki, 1989; Panbangred et al., 2000). Additionally, various isolates of *B. megaterium* can produce compounds known for their bactericidal, fungicidal, nematicidal and pesticidal activities (Huang et al., 2010; Zou et al., 2010).

In addition to *B. megaterium*, *Burkholderia* sp. a new Gram negative bacterium is one of over 40 species of the genus *Burkholderia* and which has been isolated from a soil (Agronutrition partner). The genus *Burkholderia* was known for its antibiotic (Li et al., 2002), antifungal (Mao, et al., 2006), antibacterial (Hwang et al., 2002; Kang et al., 2004) and herbicidal activities (Ludovic et al., 2007), in addition to being used in the production of large variety of enzymes such as proteases chitinases and lipases which make this genus interest for industrial purposes (Ludovic et al., 2007).

The purpose of the presented study was to determine and compare *B. megaterium* and *Burkholderia* sp., supernatants extracted with different organic solvents (cyclohexane, dichloromethane, ethyl acetate and *n*-butanol) in term of their extracted quantities, total content of phenolics, their potential for plant growth (maize and sunflower) and their anti-inflammatory (anti-5-lipoxygenase), anti-diabetic (anti-α-amylase), anti-Alzheimer (anti-acetylcholinesterase), anti-xanthine oxidase and cytotoxic activities (IGROV, OVCAR, HCT-116 and MCF-7 cell lines). Moreover and for the first time, we report here the effect of the combination of these two different Gram bacteria, which were able to inhabit the same medium, on the chemical composition, agronomic and biological activities studied in this paper.

Material and methods

Chemicals used

All chemicals used were of analytical reagent grade. All reagents were purchased from Sigma-Aldrich-Fluka (Saint-Quentin France).

Identification and culture of studied bacteria

The two studied strains have been isolated from agricultural soil samples used to grow barley and wheat, by the dilution plating technique and their ability to solubilize Tri calcium phosphate. These two strains have been characterized morphologically and identified by bacterial 16 S rRNA gene amplification and sequencing by PCR using the universal primer 1492 r and bacterial primer 27f. The resulting sequences were compared by BLAST search to The National Library of Medicine database (Bethesda, USA) giving an identification as *B. megaterium* AGN01 and *Burkholderia* sp. AGN02. The phylogenetic trees have also been established.

Bacterial strains and culture mediums were obtained from the culture collection of Agronutrition. Different microorganisms were grown over dark in a modified Bennet broth (BB) (Water 1000 mL agar 15 g/L, dextrose 10 g/L, peptone 2.5 g/l, pH 7.2,) at room temperature. A single colony of each strain from the nutrient agar plate was inoculated into the flask containing 100 ml sterile media. The flasks were then incubated under constant agitation over dark for 24 h to 72h according to the needs of the bacterium. For the mixture, a single colony of *Burkolderia sp.* and *B. megaterium* were mixed and was inoculated into the flask containing 100 ml sterile media. The bacterial mediums were then centrifuged (10,000 ×g, 20 min, 4 °C), the filtered through a 0.22 µm filter, to obtain cell free culture filtrate.

Extraction

The supernatant (5 liters) of each strain was extracted two times using solvents of increasing polarity: cyclohexane, dichloromethane, ethyl acetate and *n*-butanol All extracts were evaporated to dryness, using rotary evaporation (Rotavapor, VWR) at 30°C, to give crudes gummy extracts.

Total phenolics amount

The total phenolics amount of different extracts was determined by using the Folin-Ciocalteu method with some modification established by Kammoun El Euch et al., (2015). 100 µL of Folin-Ciocalteu reagent (0.2 N) was added to 20µL of the diluted solution of each extract prepared at a concentration of 3 mg/ml. After 5 min of incubation at room temperature, we added 80 µL of the sodium carbonate solution (75 g/L in water). Then the mixture was incubated for 15 min and the absorbances were measured at 765 nm against water blank. A standard calibration curve was plotted using gallic acid at different concentrations (0-200 mg/L). Results were expressed as mg of gallic acid equivalents (GAE)/g of dry mass (DM).

HPLC analysis

The HPLC analysis was performed in an ultimate 3000 pump- Dionex and Thermos Sepration products detectors UV-150 model. The separation was achieved on a RP-C18 column, 25 cm x 4.6 mm and particle size=5μm, at ambient temperature. Elution was performed at a flow rate of 1.2 mL/min, using a mobile consisted of acidified water (acetic acid, pH=2.65), (solvent A) and water/acetonitrile (20:80 v/v, pH=2.65). The bacterial samples were eluted by the following linear gradient: from 0.1% B to 30% B for 35 min, from 30% B to 50% B for 5 min, from 50% B to 99.9% B for 5 min and finally from 99.9% B to 0.1% B for 15 min. 20 µL of samples, prepared at the concentration of 20 µg/mL, was injected and the detection was performed at 280 nm. The identification of compounds was achieved by combination of retention time and standards spectral matching.

Anti-inflammatory activity

The anti-inflammatory activity was assessed using the spectrophotometric measurement of a conjugated diene, a product from the oxidation of linoleic acid by the enzyme 5-lipoxygenase (5-LOX) (Kammoun El Euch et al., 2015). Briefly, 20 µL of each extract (50 µg/mL in well) were mixed with 50 µL of buffer solution at ph 7.4 (Na_2HPO_4, $2H_2O$; KH_2PO_4; NaCl), 60 µL of linoleic acid and 20 µL of 5-LOX enzyme solution. After 10 min of incubation at 25°C, The absorbance was measured against a blank. Nordihydroguaiaretic acid (NDGA) was used as standard.

Anti-acetylcholinesterase activity

The inhibition of the acetylcholinesterase enzyme (AChE) was performed by Ellman's method (Kammoun El Euch et al., 2015). Briefly, 25 µL of each extract (50 µg/mL in well) were mixed with buffer A (Na_2HPO_4, $12H_2O$, (100 mM, pH=8)), 125 µL of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) solution (3 mM) prepared in 100 mM phosphate Buffer C (Na_2HPO_4, $12H_2O$, pH=7) and 25 µL of the enzyme AChE solution (1.4 U/mL) dissolved in 20 mM phosphate Buffer B (Na_2HPO_4, $12H_2O$, pH=7). After incubation for 15 min at 25°C, 25 µL of 15 mM acetylthiocholine iodide solution prepared in Buffer A was added. The mixture were then homogenized and incubated for 10 min at 25 °C. The absorbance was measured at 412 nm against a blank without extract and galanthamine was used as a positive control.

Anti xathine oxidase activity

The anti-xanthine oxidase activity was assessed using the spectrophotometric measurement of uric acid the product from xanthine oxidation (Kammoun El Euch et al., 2015). Briefly, 60 µL of phosphate buffer solution (70 nM) at pH 7.5 were mixed with 50 µL of each extract (50 µg/mg in well) and 30 µL of 0.1 U/mL xanthine enzyme solution. After 15 min agitation and incubation at 25°C, 60 µL of 150 µM xanthine solution were added to the mixture. The mixture was then incubated for 5 min; the absorbance was determined at 295 nm against a blank without the extract. Allopurinol was used as a positive control.

Anti α-amylase activity

The anti-α-amylase activity was performed by a modified procedure of Mccue and Shetty (Shalaby et al., 2014). Briefly, 50 µL of extracts (50 µg/mL in well) was mixed with 50 µL of 1 U/mL α-amylase enzyme prepared in sodium phosphate buffer solution (pH= 6.9). After 15 min pre-incubation at 25°C, 100 µL of 1% starch solution prepared in sodium buffer solution (pH= 6.9) was added. After second pre-incubation for 5 min at 25°C, 100 µL of dinitrosalicylic acid reagent was added. The obtained mixture was then incubated in boiling water for 10 min. finally, the mixture was diluted with 1 mL sodium phosphate buffer solution (pH= 6.9) and the absorbance was determined at 540 nm against a blank without the extract. Acarbose was used as positive control.

Anti-cancer activity

Cytotoxicity of each extracts was estimated against two ovarian cancer cell lines (IGROV and OVCAR), human breast cancer cell line (MCF-7) and colon cancer cell line (HCT-116) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Kammoun El Euch et al., 2015). 100 µL of each cells at a concentration of 10^4 cells/wells were distributed in 96-well plates and maintained at 37°C during 24 h in an incubator with 5% CO_2 in humidified atmosphere. Then, 100 µL of each extracts (50 µg/mg) diluted on the culture medium were added and the mixture was incubated for 48 h at 37°C. Then, the medium was removed and cells were treated with 50 µL of MTT solution, prepared in phosphate buffered saline (PBS), at a concentration of 1 mg/mL and incubated at 37°C during 40 min. After that, MTT solution was absorbed and 50 µL of DMSO were added to dissolve insoluble formazan blue crystals. Cell growth was estimated by the determination of optical density at 605 nm. Tamoxifen was used as standard.

Allelopathic Potential

Each organic extracts were tested on sunflower (*Helianthus annuus* L.) and maize (*Zea mays* L.) seeds. Seeds were surface-sterilized with 10% sodium hypochlorite solution during 6 min then washed three times with distilled water (NaCl 0.9%). Germination of *H. annuus* and *Z. mays* seedlings were evaluated in response to different organic extracts from *Burkholderia*, *B.* megaterium and the mixture of strains. In a sterilized multiwell dishes, twenty four imbibed seeds of *H. annuus* and *Z. mays* were separately placed on the supporting medium, 250 µl of each extract at a concentration of 40 µg/mL or distilled water (as control) were added for each treated seed. All treatments were conducted in triplicate. Number of germinated seeds was counted daily and percentage of seed germination was determined after 13 days for sunflower and 9 days for maize. The percentage of germination (GP) was determined using the following equation (Omezzine et al., 2013):

$$GP=(n/N) \times 100$$

Where n is the proportion of germinated seeds observed after t days and N is total number of seeds per replication.

Results and discussion

Extraction yields

The effect of the combination between *Burkholderia sp.* and *B. megaterium* on the yields of extracted molecules from the supernatant of each bacterium was investigated (Table 1). Extraction of the three supernatants (*Burkholderia* sp., *B. megaterium* and their mixture) was realized with four different solvents of increasing polarity: cyclohexane, dichloromethane, ethyl acetate and *n*-butanol. Results showed that the total quantity extracted from the *Burkholderia* sp. was higher than both *B. megaterium* and the mixture. Polar extracts have more yields than no*n*-polar extracts. The highest extracted quantity was in *n*-butanol extract made from *Burkholderia* sp. (3600 µg/mL) which was more important compared to same solvent from *B. megaterium* (1410 µg/mL) and from the mixture (1360 µg/mL). The dichloromethane and ethyl acetate extracts from *B. megaterium* have the higher extracted quantities (331 and 900 µg/mL, respectively) compared to the same extracts from *Burkholderia* sp. (62 and 580 µg/mL, respectively). After combination (mixture), the production of secondary metabolites (obtained by all solvents) was affected by the presence of *B. megaterium.* To our knowledge, no study cited in the literature concerning extraction from *B. megaterium* or *Burkholderia* sp.

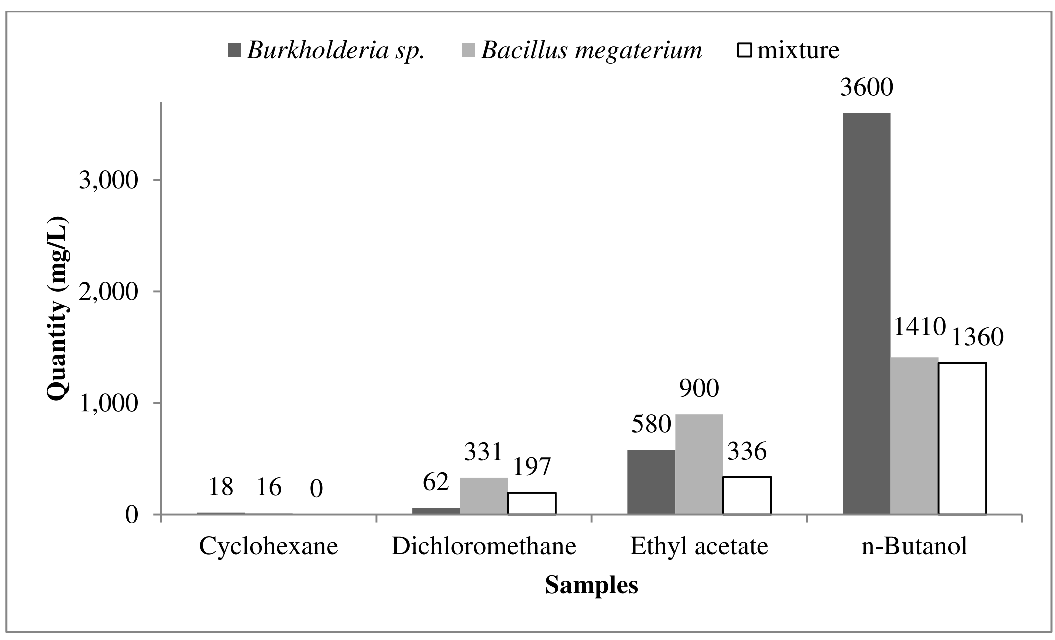

Figure 1. Extraction quantities of *Burkholderia* sp., ***Bacillus** megaterium* and the mixture extracts.

Total phenolics amount

This is the first study to investigate the influence of *Burkholderia* sp.-*B. megaterium* interaction on the production of phenolics. Results were presented in Figure 2 and showed that *n*-butanol extract from *B. megaterium* has the higher phenolics values (28.7±0.4 g GAE/kg of DM), followed by ethyl acetate extract (19.9±0.8 g GAE/kg of DM) and dichloromethane extract (19.6±1.9 g GAE/kg of DM). All extracts from *B. megaterium* have the higher phenolics amounts, except cyclohexane extract, compared to *Burkholderia* sp. and their mixture. These results indicated that *Burkholderia* sp. reduced the production of phenolics of *B. megaterium* in mixture.

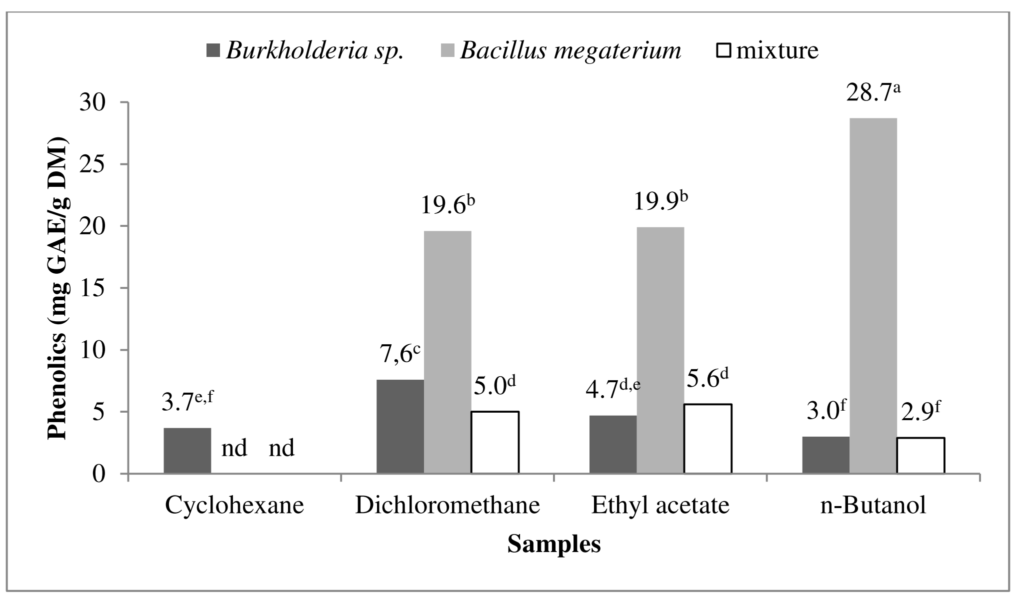

Figure 2. Total phenolic content of *Burkholderia* sp., *Bacillus megaterium* and the mixture extracts. Means with the same letters are not significantly different at P<0.05.

HPLC analysis

Reversed phase HPLC with C18 columns is one of the most popular methods developed for the determination of aromatic compounds and polyphenols of different extracts. A UV-vis (280 nm) was used for phenolic compounds. Only *n*-Butanol extracts, obtained from different strains (*Burkholderia* sp.*, B. megaterium* and the mixture of strains), were analyzed by HPLC due to their higher extracted quantities. The obtained chromatograms showed that all *n*-butanol extracts contained polar compounds. Overall, eight polar compounds with high intensity (or peak area), at 280 nm, were present in *n*-butanol extracts. Four compounds **2**, **3**, **4** and **7** (retention times; 2.05, 2.37, 2.62 and 3.11 min, respectively) were commons for all *n*-butanol extracts. However, compound **1** (1.96 min) was present only in *Burkholderia* sp. and *B. megaterium n*-butanol extracts, but, compounds **6** and **8** (retention times 3.54 and 7.68 min, respectively) were present only in *B. megaterium n*-butanol extract. In addition, *Burkholderia* sp. was able to produce another polar compound **5** (2.81 min). We notice that a great difference on the quantity of different detected polar compounds was observed. We conclude that *Burkholderia* sp.-*B. megaterium* interaction reduced the production of polar compounds detected by HPLC analysis.

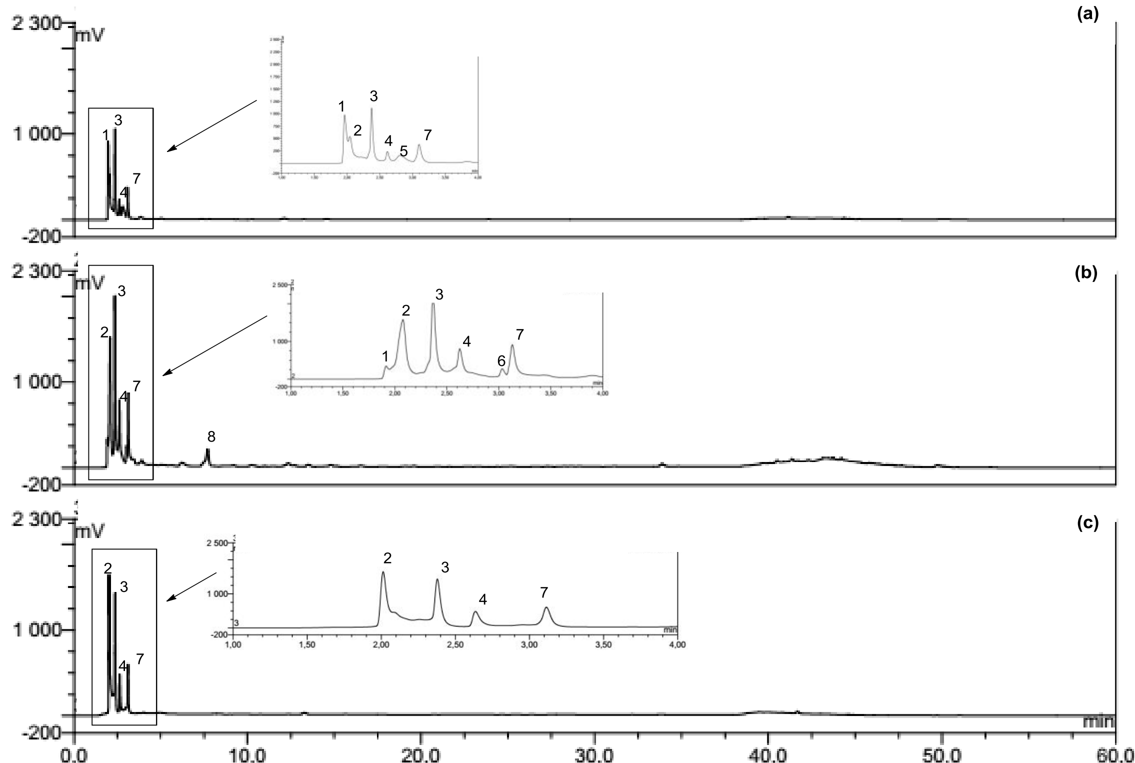

Figure 3. HPLC chromatograms of *Burkholderia* sp (a), *Bacillus megaterium* (b) and mixture (c) n-butanol extracts.

Biological activities

This is the first study that investigated the anti-inflammatory, anti-xanthine oxidase, anti-acetylcholinesterase, anti-α-amylase, anti-cancer activities and allelophatic potential of *B. megaterium* and *Burkholderia* sp.

Anti-inflammatory activity

The anti-5-LOX activity of extracts tested at 50 µg/mL were demonstrated in Table 1. All extracts of *Burkholderia* sp. inhibited the 5-LOX enzyme with slight percentage inhibition from 12.8±0.3 to 18.9±1.4%. No inhibition obtained for different extracts from *B. megaterium*. However, all extracts from the mixture of strains, except cyclohexane extract, exhibited the best activity (33.1-36.8%). These results indicate that *Burkholderia* sp. -*B. megaterium* interaction allowed the production of interesting molecules able to inhibit the 5-LOX enzyme or synergy.

Anti-acetylcholinesterase activity

The effects of different extracts, tested at 50 µg/mL, on the inhibition of the acetylcholinesterase enzyme were summarized in Table 1. Cyclohexane and dichloromethane

extracts from different strains exhibited no antiacetylcholinesterase activity. In contrast, the polar extracts exhibited a slight antiacetylcholinesterase activity with the high recorded for *Burkholderia* sp. *n*-butanol extract (IP%=14.9±0.8%). In addition, we observed the decrease of the potential of antiacetylcholinesterase, recorded for *Burkholderia* sp. *n*-butanol extract, when *Burkholderia* sp. was combined with *B. megaterium.* We can conclude that the *Burkholderia* sp.-*B. megaterium* interaction reduced the production of molecules exhibiting antiacetylcholinesterase potential or antagonism effect.

Anti-xanthine oxidase activity

Anti-xanthine oxidase activity of *Burkholderia*, *B. megaterium* and the mixture extracts was tested at 50 µg/mL. No inhibition obtained for cyclohexane extract of *Burkholderia* sp., however, a moderate activity were registered for dichloromethane, ethyl acetate and *n*-butanol extracts with IP% of 14.0±1.1, 11.3±0.7 and 6.4±0.5%, respectively. *B. megaterium* extracts exhibited more interesting inhibitory activity against xanthine oxidase with IP% values from 30.1±3.3 to 48.8±2.9% (dichloromethane extract).

Moreover, ethyl acetate and *n*-butanol extracts made from the mixture showed moderate anti-xanthine oxidase activity with IP% values of 34.8±1.9 and 17.8±1.3%, respectively but cyclohexane and dichloromethane extracts were inactive. We can concluded that an antagonistic effect on the anti-xanthine oxidase activity was seen after combination of *B. megaterium* with *Burkholderia* sp.

Table 1. Anti-inflammatory (5-lipoxygenase), anti-acetylcholinesterase, antidiabetic (α-amylase) and anti-xanthine oxidase activities of *Burkholderia* sp., *B. megaterium* and mixture extracts (50 µg/mL).

	sample	Anti-inflammatory activity (%)	Anti-acetylcholinesterase activity (%)	Anti-xanthine oxidase activity (%)	anti-α-amylase activity (%)
B. megaterium	Cyclohexane	na	na	46.8±2.1[a]	45.9±0.9[b,c,d]
	Dichloromethane	na	na	48.8±2.9[a]	33.7±0.9[c,d]
	Ethyl acetate	na	3.6±0.8[d,e]	30.1±3.3[d]	58.0±2.4[b]
	n-butanol	na	3.5±1.8[e]	37.4±3.1[b,c]	27.2±4.4[d]
***Burkholderia* sp.**	Cyclohexane	12.8±0.3[f]	na	na	23.0±1.8[d]
	Dichloromethane	16.0±1.2[e]	na	14.0±1.1[e,f]	33.0±0.4[c,d]
	Ethyl acetate	18.9±1.4[d]	na	11.3±0.7[e,f]	5.8±0.3[e]
	n-butanol	18.1±0.9[d]	14.9±0.8[b]	6.4±0.5[f]	2.5±0.3[e]
Mixture	Cyclohexane	na	na	na	na
	Dichloromethane	33.1±0.7[c]	na	na	73.5±4.7[a]
	Ethyl acetate	34.9±0.8[c]	4.5±0.4[d]	34.8±1.9[c,d]	34.5±0.4[b,c,d]
	n-butanol	36.8±1.2[b]	6.6±0.3[c]	17.8±1.3[e]	45.8±0.1[b,c,d]
NDGA (2.0 µg/mL)		53.1±0.1[a]	-	-	-
Galanthamine (1.0 µg/mL)		-	48.3±0.1[a]	-	-
Allopurinol (1.0 µg/mL)		-	-	46.2±0.2 [a,b]	-
Acarbose (50 µg/mL)		-	-	-	48.4±0.9[b,c]

NDGA: nordihydroguaiaretic acid. na: not active. Data are the means of three independent experiments±standard deviations (n=3). Means with the same letters in a column are not significantly different at P<0.05.

Anti-α-amylase activity

The α-amylase inhibition by *Burkholderia*, *B. megaterium* and the mixture extracts were demonstrated in Table 1. All extracts of *Burkholderia* sp. inhibited the α-amylase enzyme with slightly percentage from 2.5±0.3 (*n*-butanol extract) to 33.0±1.3% (dichloromethane extract).

For *B. megaterium*, the results showed that all extracts inhibited significantly the α-amylase enzyme. Therefore, the ethyl acetate extract was significantly more active (IP%=58.0±2.4%), followed by cyclohexane extract (IP%=45.9±0.9%), dichloromethane extract (IP%=33.7±0.9%) and *n*-butanol extract (IP%=27.2±4.4%). The ethyl acetate extract exerted very strong anti-α-amylase activity which was highly comparable to acarbose reference. These results proved the presence in extract interesting compound(s) more potent compared to acarbose.

When *B. megaterium* was combined with *Burkholderia* sp., the anti-α-amylase activity of cyclohexane and ethyl acetate extracts decreased, contrary to dichloromethane and *n*-butanol extracts which exhibited more powerful activity especially dichloromethane (IP%=73.5±4.7%). These results proved the presence in extract interesting and more potent anti-α-amylase compared to acarbose.

Anti-cancer activity

Cytotoxicity evaluation against four cell lines (OVCAR, IGROV, MCF-7 and HCT-116 cell lines) were demonstrated at 50 µg/mL (Table 2). Extracts of *Burkholderia* sp. showed slightly anti-cancer activity against all cells (ranging from 8.9±1.3 to 40.3±4.8%).

All *B. megaterium* extracts were found to be inactive against tested cell lines, except cyclohexane extract exhibited an exceptional and interesting cytotoxic activity against all tested cell lines (IP%=60.04±14.0, 96.5±2.5, 98.3±0.8 and 99.2±1.2% against OVCAR, IGROV, MCF-7 and HCT-116 cell lines, respectively).

Mixture extracts, made from the combination of the two strains, were being slightly active against all cell lines tested in this study with a percentage inhibition ranging from 8.9±1.3 to 32.1±4.4%. However and in contrast to *B. megaterium* cyclohexane extract which was the most active extract, mixture cyclohexane extract was inactive towards all cell lines. This result showed that combination of the two strains reduced the production of molecules exhibiting antiacetylcholinesterase potential or antagonism effect.

Table 2. Cytotoxic activity of different extracts obtained by *Burkholderia* sp., *B. megaterium* and the mixture (tested at 50 µg/mL).

	Extract	**Anti-cancer activity (%)**			
		MCF-7	**IGROV**	**OVCAR**	**HCT-116**
B. megaterium	Cyclohexane	98.3±0.8[a]	96.5±2.5[a]	60.04±14.0[a]	99.2±1.2[a]
	Dichloromethane	37.2±4.9[b]	7.5±4.7[e,f]	na	na
	Ethyl acetate	na	na	na	2.9±1.2[e]
	n-butanol	na	na	8.61±2.1[b,c,d,e]	na
***Burkholderia* sp**	Cyclohexane	na	28.6±1.1[b]	17.3±1.8[b,c,d]	31.6±6.3[b]
	Dichloromethane	25.2±1.6[b,c]	14.9±2.2[c,d,e]	na	17.1±2.8[c,d]
	Ethyl acetate	40.3±4.8[b]	22.1±2.8[b,c]	18.4±4.3[b,c]	8.9±1.3[d,e]
	n-butanol	24.8±9.9[b,c]	20.7±2.7[b,c,d]	na	9.6±2.4[d,e]
Mixture	Cyclohexane	na	na	na	na
	Dichloromethane	32.1±4.4[b,c]	9.0±1.4[d,e,f]	26.6±14.9[b]	9.3±2.8[d,e]
	Ethyl acetate	30.8±4.3[b,c]	21.7±5.1[b,c,d]	15.7±6.5[b,c,d,e]	22.9±1.3[b,c]
	n-butanol	19.0±3.5[c]	11.5±8.7[c,d,e]	15.9±5.7[b,c,d,e]	9.4±2.4[d,e]
Tamoxifen (0.2 µg/mL)		47.2±4.3[b]	42.2±1.3[b]	61.8±4.2[a]	48.6±2.3[b]

na: not active. Data are the means of three independent experiments±standard deviations (n=3). Means with the same letters in a column are not significantly different at $P<0.05$.

Allelopathic Potential

Bacteria and fungus were able to produce allelochemicals playing an important role in the growth and/or resistance of plants. The secondary metabolites can vary among species of bacteria. This study focuses on whether extracts from a Gram-negative bacterium such as *Burkholderia* sp., Gram positive bacterium such as *B. megaterium* and mixture of the two bacteria, can modulate seed germination. The extracts were tested, for the first time, against seeds of sunflower (*Helianthus annuus* L.) and maize (*Zea mays* L.). The results show that extracts from the different mediums varied in their toxicity, depending on the nature of bacterium and the nature of solvents used for extraction. Cyclohexane extracts have not been tested because of the low quantity.

For sunflower seeds, the results showed that the greater percentage of germination was recorded with ethyl acetate and *n*-butanol extracts from *Burkholderia* strain (% Germination=97.92%) followed by *n*-butanol and dichloromethane extracts made from *B. megaterium* and extract made from the mixture with the same% Germination=97.22%. In contrast and in presence of dichloromethane extract made from *Burkholderia* supernatant, Figure 3 shows that sunflower germination was inhibited unlike its behaviour with control (% Germination=77.08%) which indicate that this extract was toxic for germination.

For maize seeds, the germination was highly affected by different extracts. Indeed, ethyl acetate extract made from *B. megaterium* and *n*-butanol extract made from *Burkholderia* was the most toxic which significantly delayed germination of maize seeds (% Germination=62.50% and 77.08% respectively, unlike its behaviour with control). For the rest of extracts, the percentage of germination went from 85.42% to 97.92% which indicate that these extracts improved germination.

Similar to our results, many researchers reported that different rhizobacteria such as *B. subtilis, B. lichenoformis* and *Pseudomonas* spp. were able to stimulate germination and seedling growth of many plants (maize, sunflower, tomato...) (Lim and Kim, 2009; Noumavo et al., 2013; Widnyana and Javandira, 2016) however our results are the first that discuss the importance of *B. megaterium* AGN01, *Burkholderia* sp. AGN02 and their mixture on the production of allelochimicals able to promote seed germination of maize and sunflower seeds.

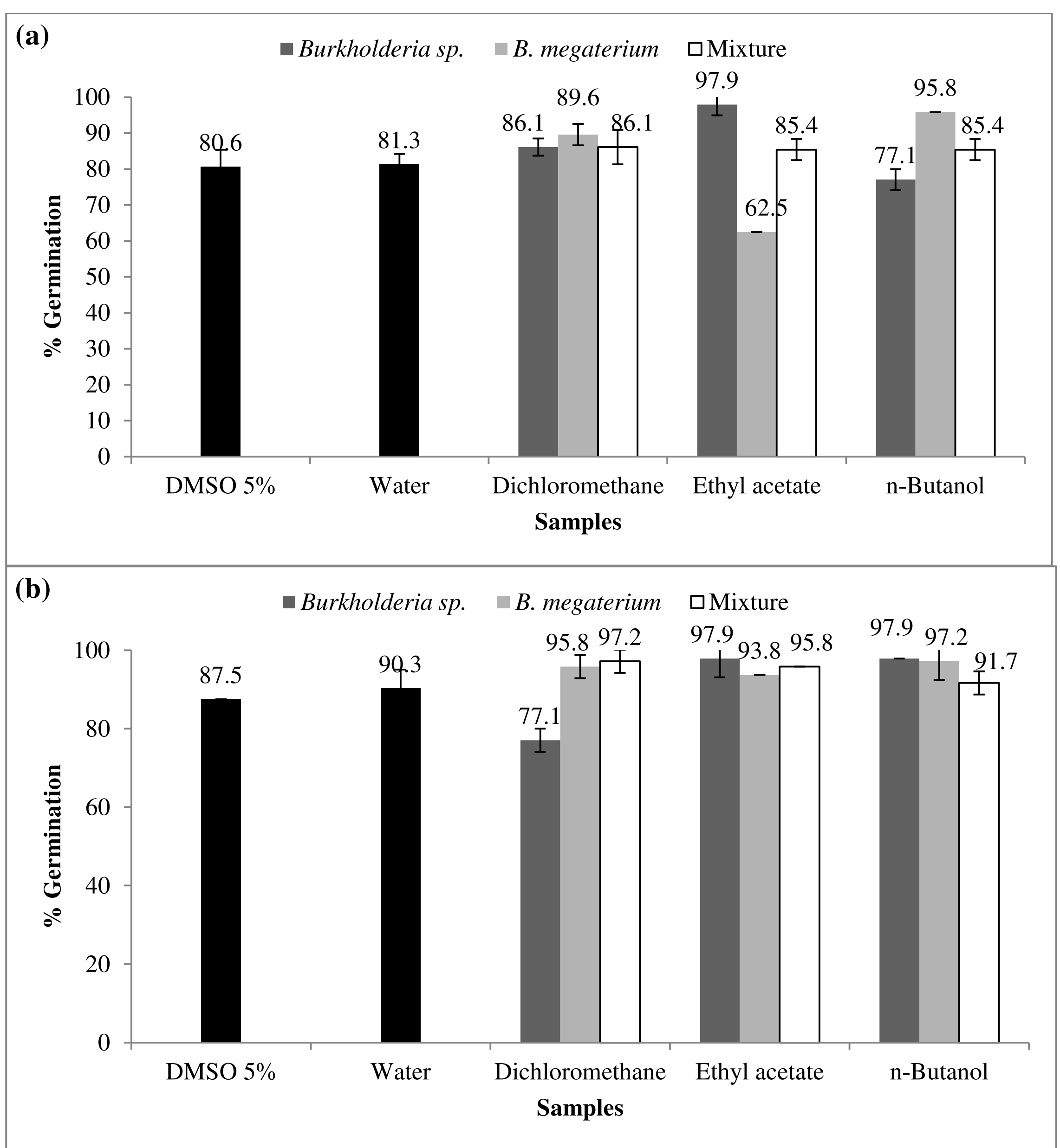

Figure 4. Allelophatic effects of different extracts of *Burkholderia* sp. *Bacillus megaterium* and mixture on percentage of germination of (a) *Zea mays* and (b) *Helianthus annuus*. Water and DMSO (5%) were used as control

Conclusion

The present study highlighted the significant influence of combination of two bacteria on the phenolic contents and on biological activities. Both bacteria *n*-butanol extracts analysed separately or mixed together showed the ability to produce aromatic and phenolic compounds with different proportion. In addition, both bacteria extracts tested separately are endowed with moderate anti-diabetic activity, while, mixture extracts exhibited a powerful anti-diabetic activity comparable to the pure compound acarbose. The absence of cytotoxic property in

mixture extracts could highlight the idea of using these extracts in food industry, essentially being hypoglycemic agents as it exhibited powerful anti-α-amylase activity.

Moreover, obtained results showed that *B. megaterium* extracts, especially cyclohexane extract, exerted more interesting inhibitory activities against different cancer cells lines and XOD enzyme. Further studies are in progress to target any specific interesting molecules which may be responsible for the observed biological activities and open so a new horizon about *Burkholderia* and *B. megaterium* possible utilizations in several fields such as food, medicine and pharmaceutical industries.

References

Carvalho, A., P., G. M. Ventura, C. B. Pereira, R. S. Leao, T. W. Folescu, L. Higa, L. M. Teixeira, M. C. Plotkowski, V. L. Merquior, R. M. Albano, E. A. Marques. 2007. *Burkholderia cenocepacia*, *B. multivorans*, *B. ambifaria* and *B. vietnamiensis* isolates from cystic fibrosis patients have different profiles of exoenzyme production. *APMIS*. 115: 311-318.

De Bary, A. 1884. *Vergleichende Morphologie und Biologie der Pilze, Mycetozoen und Bakterien*. Leipzig, Germany: Wilhelm Engelmann.

Huang, Y., Xu, C., K., Ma, L., Zhang, K., Q., Duan, C., Q., Mo, M., H. 2010. Characterisation of volatiles produced from *Bacillus megaterium* YFM3.25 and their nematicidal activity against *Meloidogyne incognita*. *Eur J Plant Pathol*. 126, 417-422

Hwang, J., Chilton, W. S., Benson, D., M. 2002. Pyrrolnitrin production by *Burkholderia cepacia* and biocontrol of *Rhizoctonia* stem rot of poinsettia. *Biol.Control*. 25, 56-63.

Kammoun El Euch, S., Bouajila, J., Bouzouita, N. 2015. Chemical composition, biological and cytotoxic activities of *Cistus salviifolius* flower buds and leaves extracts. *Ind Crops Prod.* 76, 1100-1105.

Kang, J., G., Shin, S., Y., Kim, M., J., Bajpai, V. Maheshwari, D., K., Kang, S., C. 2004. Isolation and antifungal activities of 2-hydroxymethyl-chroman-4-one produced by *Burkholderia* sp. Mssp. *J. Antibiot.* 57, 726-731.

Li, W., D. P. Roberts, P. D. Dery, S. L. F. Meyer, S. Lohrke, R. D. Lumsden, K. P. Hebbar. 2002. Broad spectrum antibiotic activity and disease suppression by the potential biocontrol agent *Burkholderia ambifaria* BC-F. *Crop Prot. J*. 21: 129 -135.

Lim, J.H., Kim, S.D. 2009. Synergistic plant growth promotion by the indigenous auxinsproducing PGPR *Bacillus subtilis* AH18 and *Bacillus Licheniforims* K11. *J Korean Soc Appl Biol Chem* 52, 531-538.

Ludovic, V., Groleau, M., C., Dekimpe, V., Déziel, E. 2007. *Burkholderia* Diversity and Versatility: An Inventory of the Extracellular Products. *J. Microbiol. Biotechnol.* 17, 1407-1429.

Mao, S., S. J. Lee, H. Hwangbo, Y. W. Kim, K. H. Park, G. S. Cha, R. D. Park, K. Y. Kim. 2006. Isolation and characterization of antifungal substances from *Burkholderia* sp. culture broth. *Curr. Microbiol.* 53, 358 -364.

Noumavo. P.A, Kochoni, E., Didagbé, Y.O., Adjanohoun, A., Allagbé, M., Sikirou, R., Gachomo, E.W., Kotchoni, S.O., Baba-Moussa, L. 2013. Effect of different plant growth promoting rhizobacteria on maize seed germination and seedling development. *Amer J Plant Sci* 4, 1013-1021.

Panbangred, W., Weeradechapon, K., Udomvaraphant, S., Fujiyama, K., Meevootisom, V. 2000. High expression of the penicillin G acylase gene (*pac*) from *Bacillus megaterium* UN1 in its own *pac* minus mutant. *J Appl Microbiol***.** 89, 152-157.

Shalaby, N.M.M., Abd-Alla, H.I., Aly, H.F., Albalawy, M.A., Shaker, K.H., Bouajila, J. 2014. Preliminary *in vitro* and *in vivo* evaluation of antidiabetic activity of *Ducrosia anethifolia* Boiss and its linear furanocoumarins. *BioMed. Res. Int.* ID 480545, 1-13.

Takasaki, Y. 1989. Novel maltose-producing amylase from *Bacillus megaterium* G-2. *Agr Biol Chem.* 53, 341-347.

Widnyana, I.K, Javandira, C. 2016. Activities *Pseudomonas* spp. and *Bacillus* sp. to stimulate germination and seedling growth of tomato plants. *Agr and Agr Sci Procedia.* 9, 419-423.

Zou, C., Li, Z., Yu, D. 2010. *Bacillus megaterium* Strain XTBG34 Promotes Plant Growth by Producing 2-Pentylfuran. *J. Microbiol..* 4, 460-466.

Conclusion

L'objectif de ce présent chapitre de thèse, était d'étudier l'influence des intéractions bactériennes sur la composition chimique (composés phénoliques, HPLC et composés volatiles) de *B. megaterium* AGN01 *Burkholderia* sp.AGN02 et leur mélange, ainsi que son influence sur les activités biologiques. Pour se faire, nous avons développé plusieurs études dont le bilan de différents points abordés au cours de ce chapitre peut être résumé comme suit :

- Nous avons mis en évidence l'influence notable de la combinaison de deux bactéries sur le contenu phénolique et sur les activités biologiques. Les deux extraits de bactéries du *n*-butanol analysés séparément ou en mélange ont montré l'aptitude à produire des composés aromatiques et phénoliques avec des proportions différentes. De plus, les extraits de deux bactéries testés séparément sont dotés d'une activité anti-diabétique modérée, tandis que, les extraits de mélange dotés d'une activité anti-diabétique significative puissante comparable à l'acarbose (référence-composé pur). Par ailleurs, les résultats obtenus ont montré que les extraits de B. *megaterium*, en particulier l'extrait de cyclohexane, est doté des activités cytotoxique et inhibitrice de l'enzyme XOD très intéressantes.
- La composition chimique des métabolites des souches ainsi que leur mélange a été étudiée en determinant leurs profils volatils (extraits préparés : le cyclohexane, le dichlorométhane, l'acétate d'éthyle et le *n*-butanol) utilisant la GC-HRMS et la réaction de dérivatisation. On a constaté que plus de soixante composés organiques volatils ont été identifiés à partir des différents extraits appartenant à différentes classes de composé chimique tel que les composés soufrés, les furanes, les hydrocarbures, les cétones, les acides carboxyliques, les aldéhydes, les alcools, les esters et les dicétopipérazines. L'analyse par GC-HRMS nous a permis d'identifier plus que cinquante composés dont *N*-butylbenzenesulfonamide, triacontane, octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate et (*E*)-5-chloro-3-(hydroxyimino)indolin-2-one cités pour la premiere fois dans des bactéries. Les résultats obtenus ont indiqué que la combinaison de *Burkholderia* et *B. megaterium* a influencé la production de mélanges de matières volatiles émises par les deux souches.

Chapitre IV : Etude de l'effet du stockage sur la composition chimique de Burkholderia sp. et Bacillus megaterium et sur les activités biologiques

Introduction

Les Bacilles et les proteobacteries du genre *Burkholderia* sont parmi les meilleurs candidats pour la production des métabolites secondaires biologiquement actifs. En effet, ces deux genres bactériens sont à l'origine de nombreux agents bioactifs et qui sont utilisés pour leurs propriétés fonctionnelles dans les domaines pharmaceutiques ainsi qu'agronomique. Parmi ces agents, on peut citer les antimicrobiens, les antifongiques, les antibiotiques, les anticancéreux, etc. Plusieurs travaux ont été menés pour augmenter la production de métabolites secondaires d'intérêts biologique à partir de ces deux genres bactériens. Il a été démontré, à coté de la nature de bactérie, l'importance majeure et l'implication de l'influence des conditions de culture sans oublier les conditions de stockage de la bactérie productrice sur la production du ou des métabolites recherchés.

Dans ce chapitre, notre intérêt s'est porté à l'étude de l'évolution de la production des métabolites secondaires à intérêt biologique suite à un stockage à longue durée. Les résultats obtenus ainsi que leurs discussions ont été présentés en deux parties comme suit :

- La première partie s'intéresse à l'étude de l'impact de stockage sur la production des métabolites volatils microbiens à partir des mêmes souches bactériennes. Au cours de cette partie, on a eu recours à la GC-SM ainsi qu'a la silylation de différent extraits pour étudier l'effet de stockage des souches bactériennes (*Burkholderia* sp., *B. megaterium* et le mélange de deux souches) sur la production des métabolites secondaires volatils bactériens. Cette partie de thèse fait l'objet d'une publication qui est actuellemnt soumise «*Antonie van Leeuwenhoek*».
- La deuxième partie a été consacrée à l'étude de l'effet stockage sur la composition chimique (polyphénols Totaux et analyse HPLC) et les activités biologiques (l'activité anti-inflammatoire (anti-5-lipoxygénase), activité anti-xanthine oxydase, l'activité anti-diabétique (anti-α-amylae), la cytotoxicité (MCF-7, HCT-116 et OVCAR) de différents extraits obtenu à partir de ces deux mêmes souches bactériennes. Cette deuxième partie fait l'objet d'une publication qui est actuellemnt soumise «*Saudi Pharmaceutical Journal*».

Partie A : Impact of long-term storage on the production of microbial volatiles compounds by *Bacillus megaterium* AGN01 and *Burkholderia* sp. AGN02

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Abstract

Our study is in line with the analyze of the influence of storage conditions on the production of microbial compounds (Vocs) by different bacteria. So the aim was to characterize the effect of storage conditions of two different strains isolated from different soil samples (*Burkholderia* sp. AGN02 and *Bacillus megaterium* AGN01). Thirty six compounds were identified from different extracts (cyclohexane, dichloromethane, ethyl acetate and *n*-butanol) using gas chromatography-high resolution mass spectrometry and silylation reaction. According to the obtained results, a large variety of compound classes such as hydrocarbons, alcohols, aldehydes, esters and diketopiperazines were identified including 1,3,5-trimethyl-2-octadecylcyclohexane **20**, *N*,*N*-diethyl-10,13-dimethyl-17-(6-methylheptan-2-yl)-4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-amine **23** and 6-((*E*)-4-((*E*)-2-(5-hydroxy-2-methylenecyclohexylidene)ethylidene)-7a-methyl-octahydro-1*H*-inden-1-yl)-2-methylheptane-2,3-diol **30** reported for the first time by bacteria. In addition, we found that the response of each bacterium to long-term storage was different; therefore, storage for long periods of time decreased the production of volatiles by *Burkholderia* sp., in contrast, the production of volatiles increased for *B. megaterium* and the mixture.

Keywords

Burkholderia sp., *Bacillus megaterium*, MVOCs, silylation, alcohols, hydrocarbons, diketopiperazines

Introduction

Interest in microorganisms as a source of volatile organic compounds known for their intervention in mediating interactions between bacteria and other organisms has increased worldwide, particularly in the search of new bacterial volatiles and their effects on bacteria, fungi, and plant. The production of volatile compounds by bacteria are regulated and depended on their taxonomic identity, environments, enzyme induction, their growth stage, as well as on the culture media (Schulz and Dickschat, 2007).

B. megaterium, the largest known *Bacillus* species, has been found in a variety of niches, such as soil, plants, seawater and sediment (Vary et al., 2007). In addition, *B. megaterium* specie has been found able to produce large variety of volatile compounds such as diketopiperazines, furan containing compounds, alcohols, sulphur containing compounds as well as ketones (Huang et al., 2010; Zou et al., 2010). It produces also a large variety of organic volatile compounds that promote plant growth (Zou et al., 2010).

In addition, *Burkholderia* species, known for their ecological versatility, has been found to inhabit diverse niches such as soil, plants, water, insects and even infected humans (Ludovic et al., 2007). It was also shown that some *Burkholderia* species, that promote plant growth, were able to secrete a variety of volatile organic compounds such as diketopiperazines, sulphur containing compounds, amino containing compounds, alcohols, ketones, aldehydes as well as carboxylic acids (Wang et al., 2010).

This study aims to valorize the impact of microbial storage on volatile compounds produced by bacteria co-existed in agricultural soil (*Bacillus megaterium* AGN01, *Burkholderia* sp. AGN02 and their combined mixture) by comparing mediums before and after storage (three months).

Materials and methods

Chemicals

All chemicals used were of analytical reagent grade. All reagents were purchased from Sigma-Aldrich-Fluka (Saint-Quentin France)

Identification and culture of studied bacteria

The two studied strains have been isolated from samples of agricultural soils used to grow barley and wheat, by the dilution plating technique and their ability to solubilize tri-calcium phosphate. These two strains have been characterized morphologically and identified by sequencing of the bacterial 16 S rRNA gene after PCR amplification using the universal

bacterial primers 27f and 1492r. The resulting sequences were compared to NCBI Nucleotides database by BLAST giving an identification as *Bacillus megaterium* (AGN01) and *Burkholderia* sp. (AGN02). The phylogenetic trees have also been established.
Bacterial strains and culture media were obtained from the culture collection of Agronutrition (Agronutrition, Labège France). *Burkholderia* sp. AGN02 was grown in a modified Bennett broth (water 1000mL; glycerol 6.3 g/L; peptone from soybean 2.5 g/L; yeast extract 1.5 g/L; pH 7.2) at 30°C during 24h under agitation. *B. megaterium* AGN01 was grown in a modified Bennett broth (water 1000mL; dextrose 6.3 g/L; peptone from soybean 2.5 g/L; yeast extract 1.5 g/L; pH 7.2) at 30°C during 24h under agitation. A total volume of 5000 mL of bacterial cultures was obtained and split in two. Half of this bacterial culture was centrifuged at 10^4 g for 20 min at 4°C and filtered through a 0.22 µm filter, to obtain cell free culture filtrate stored at 4°C during seven days before extraction. The second part of the bacterial culture was stored at 4°C during three months and then filtered through a 0.22 µm filter before extraction.

Extraction

For extraction, each filtered supernatant were extracted using different solvents of croissant polarity: cyclohexane (cyclo), dichloromethane (DCM), ethyl acetate (EtOAc) and *n*-butanol (BuOH). All obtained organic extracts were evaporated and concentrated by rotary evaporation under vacuum at 30°C.

Gas chromatography-mass spectrometry

Gas chromatography- mass spectrometry (GC-MS) analyses of each sample were performed with a GC coupled to MS (GCT 1er Waters) and piloted by Mass Lynx.
For analysis: each sample (before and after derivatization) was dissolved in their solvent. One microliter of sample was injected in the split mode (split ratio: 10). Inlet pressure was 1 kPa and helium was used as carrier gas at an on-column flow of 1 mL/min. The gas chromatograph was programmed as follows: isothermal at 60°C for 5 min, temperature gradient 60-270°C at 15°C/min, isothermal at 270°C for 6 min, then a second gradient was applied to 300°C at 50°C/min and finally isothermal at 300°C for 4.5 min. Total analysis time was 30 minutes. High resolution mass spectrometer was adjusted for an emission current of 663 µA and electron multiplier voltage 70 eV. Trap temperature was 250°C and that of the transfer line was 300°C.Mass scanning was performed from 40 to 650 amu.

The volatile compounds were identified by the determination of their formula using Mass Lynx and by comparison with those cited in NIST 11 spectrum library.

Derivatization method

Using a modified method described by Wenclawiak et al. (1993). Briefly, 150 µL Pierce BSTFA + 1% TMCS was added to extracts (1 mL, 5 mg/mL) in THF anhydrous, and mixed in a 2 mL vial. Then, the mixture was aerated with bubbling using nitrogen and shacked for 30 seconds. The reaction mixture was maintained at 40°C for 15 min. 1 µL of each derivatized solution was finally injected into the GC-MS and analyzed as described in previous section.

Results and discussion

To our knowledge, this work is the first report on the influence of long-term storage on the chemical composition of *B. megaterium*, *Burkholderia* sp. as well as their mixture.

GC-MS chemical composition without derivatization

By GC-MS analysis, volatile compounds from different extracts were identified (Table 2) belonging to varieties of compound classes such as hydrocarbons, alcohols, ketones, aldehydes, carboxylic acids, esters, alkaloid compounds and diketopiperazines. Overall, thirty one compounds were identified. Therefore, this is the first report on the production by bacteria of 1,3,5-trimethyl-2-octadecylcyclohexane **20**, *N,N*-diethyl-10,13-dimethyl-17-(6-methylheptan-2-yl)-4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetrade-cahydro-1H-cyclopenta[a]phenanthren-3-amine **23** and 6-((*E*)-4-((*E*)-2-(5-hydroxy-2-methylenecyclohexylidene)ethylidene)-7*a*-methyloctahydro-1*H*-inden-1-yl)-2-methylheptane-2,3-diol **30**. In addition, there were no reports on the production by bacteria of the species *Bacillus* and/or *Burkholderia* of 3,4-dihydroxy-3,4-dimethylhexane-2,5-dione **3**, 1,1'-bi(cyclohexane) **5**, cyclotetradecane **8**, 2,3,5,6-tetramethyl-1,4-dioxane-2,5-diol **9**, (*E*)-heptadec-15-enal **14**, 3,7,11-trimethyldodecan-1-ol **24** and 2,2'-methylenediphenol **26**. Finally, all the rest of identified compounds were reported here for the first time in *B. megaterium* and *Burkholderia* sp. but reported from other *Bacillus* and *Burkholderia* species (Kanchiswamy et al., 2015; Bitas et al., 2013; Uta et al., 2012; Kai et al., 2009).

It can be noticed that two of these compounds are sulfur-containing and ether oxide ones and were detected only in the cyclohexane extract from immediately treated *Burkholerdia* sp. strain and were identified as 1,2-didodecyldisulfane **31** and 1-(octyloxy)octane **12**. Beside

these class of compounds, a saturated and unsaturated hydrocarbons such as 3,4-dimethylhex-1-ene **1**, 1,1'-bi(cyclohexane) **5** and 2,6,10-trimethyltetradecane **13** were only detected on immediately treated *Burkholderia* sp. cyclohexane extract, in contrast, hydrocarbons such as cyclotetradecane **8** and (*E*)-henicos-10-ene **18** were common for both immediately treated and stored *Burkholderia* sp. ethyl acetate extract. These results showed that the number of sulfur compounds, ether oxide and hydrocarbons detected in stored *Burkholderia* sp. decreased, so we can conclude that their production by the *Burkholderia* sp. strain has been affected by the long-term storage. Several alcohols like glycerol **6** (BuOH), 2,4-di-*tert*-butylphenol **10** (cyclo and EtOAc), hexadecan-1-ol **11** (EtOAc) and heptadecan-1-ol **22** (EtOAc) were common for both immediately treated and stored *Burkholderia* sp. However, alcohols like 2-hexyldecan-1-ol **7** and 6-((*E*)-4-((*E*)-2-(5-hydroxy-2-methylenecyclohexylidene)ethylidene)-7a-methyloctahydro-1H-inden-1-yl)-2-methyl-heptane-2,3-diol **30** were only detected in immediately treated *Burkholderia* sp. cyclohexane extract. Amazingly we observed that the decrease of detected of alcohols after storage was associated with parallel increases in number of detected carbonyl containing compounds (carboxylic acid and ester); therefore, (*E*)-tetradec-9-enoic acid **21** and dioctyl adipate **25** (DCM) were present only in stored *Burkholderia* sp. These results showed that *Burkholderia* sp. was able to oxidize alcohols into carboxylic acids and esters. We should notice that alcohols and carboxylic acids were known for their interference with plant and stimulation of plant growth (Kai et al., 2010; Blom et al., 2011).

Another chemical class of compounds were the diketopiperazines, which are the smallest cyclic peptides known and representing one major group of organic volatiles emitted by bacteria (Schulz and Dickschat 2007). Moreover, some diketopiperazines were eluted in column chromatography with different retention times (same mass spectra) and we supposed that one peak is for the *Levogyre* isomer and the other peak are for the *Dextrogyre* isomer (Jian-Hua et al., 2010). 3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (**17** and **19**) was produced by all mediums before and after storage (cyclohexane and dichloromethane extracts from *Burkholderia sp, B. megaterium* and the mixture of strains), however, the 3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione **29** was detected only in both immediately treated and stored mixture dichloromethane extract. According to previous report (Jian-Hua et al., 2010), these cyclic peptides can be generated by the complex medium used to culture our bacteria including yeasts. A negative control test allowed us to detect effectively a small amount of 3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (**17** and **19**) and 3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione **29**. This provide evidence that 3-

isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (**17** and **19**) and 3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione **29** (mixture) not only produced by our bacteria but also by the culture medium.

For *B. megaterium*, results showed that this strain was able to produce different kind of volatiles and overall twelve compounds were detected in the different *B. megaterium* extracts, except the *n*-butanol extract which didn't contain volatiles. Nine compounds were common to both immediately treated and stored *B. megaterium* strains (DCM and/or EtOAc extracts): 2-ethylbutanoic acid **2**, cyclotetradecane **8**, 2,4-di-*tert*-butylphenol **10**, hexadecan-1-ol **11**, (*E*)-heptadec-15-enal **14**, 3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (*Dextrogyre* and *Levogyre* isomers) (**17** and **19**), heptadecan-1-ol **22** and dioctyl adipate **25**. While, three other volatiles were produced only by stored *B. megaterium* and identified as 3,4-dihydroxy-3,4-dimethylhexane-2,5-dione **3** (cyclo and DCM), ethyl butyrate **4** (EtOAc) and palmitic acid **28** (cyclo). These results showed that the number of volatile compounds, in stored *B. megaterium* increased, so we can conclude that their production by *B. megaterium* is a response to the storage for long periods of time.

When *Burkholderia* sp. was combined with *B. megaterium*, the number of identified volatiles decreased from twenty-one detected in *Burkhlderia* strain to sixteen compounds detected in the mixture. Seven volatiles, detected in the both immediately treated and stored mixture of strains, were detected in both separately strains: cyclotetradecane **8** (EtOAc), 2,4-di-*tert*-butylphenol **10** (EtOAc), hexadecan-1-ol **11** (EtOAc), (*E*)-heptadec-15-enal **14** (EtOAc), 3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione (*Levogyre* and *dextrogyre*) **17** and **19** (DCM), and heptadecan-1-ol **22** (EtOAc). In addition, results showed that two products (3,4-dimethylhex-1-ene **1** and 1,1'-bi(cyclohexane) **5**) were commons to cyclohexane extract from immediately treated *Burkholderia* sp. and both immediately treated and stored mixture of strains which indicate that probably *Burkholderia* sp. is the responsible of its productions. In other hand, 2-ethylbutanoic acid **2** identified in *B. megaterium* ethyl acetate extract extracts was present in both immediately treated and stored mixture extracts. This product was probably produced by *B. megaterium*. Amazingly, we should notice that the six other detected compounds presented only in mixture extracts and led us to conclude that the production of these compounds is a result of response to *Burkholderia* sp.-*B. megaterium* interaction. Only two of the six compounds were commons to both immediately treated and stored mixture and identified as; 3,7,11-trimethyldodecan-1-ol **24** (EtOAc) and 3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione **29** (DCM). Furthermore, immediately treated mixture was unable to produce other volatiles, in contrast, stored one showed the ability to produce more volatile

compounds identified as 2,3,5,6-tetramethyl-1,4-dioxane-2,5- diol **9** (DCM), ethyl pentadecanoate **16** (cyclo), 1,3,5-trimethyl-2-octadecylcyclohexane **20** (BuOH) and *N*,*N*-diethyl-10,13-dimethyl-17-(6-methylheptan-2-yl)-4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-amine **23** (BuOH).

Table 1. Volatile compounds identified in different extracts of *Burkholderia* sp., *Bacillus megaterium* and combined strains.

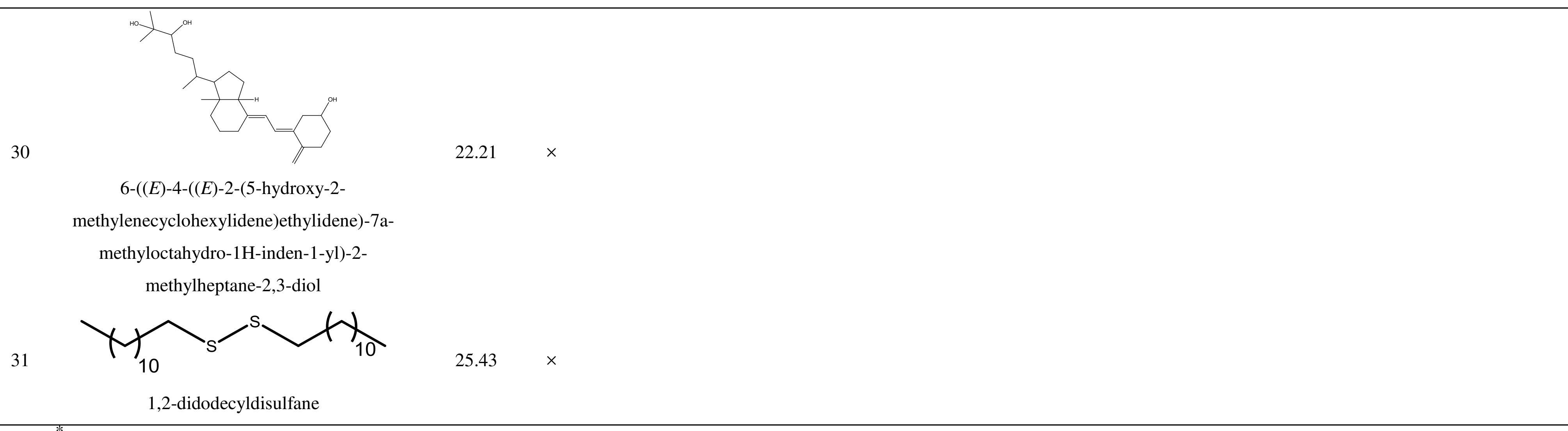
N°	Compounds	r.t. (min)	***Burkholderia* sp.**								***Bacillus megaterium***						**Mixture**							
			cyclohexane		dichloromethane		Ethyl acetate		*n*-Butanol		cyclohexane		dichloromethane		Ethyl acetate		cyclohexane		dichloromethane		Ethyl acetate		*n*-Butanol	
			I.T.	Stored	I.T.	Stored	I.T.	Stored	I.T.	Stored	I.T.	Stored	I.T.	Old	I.T.	Old	I.T.	Old	I.T.	Old	I.T.	Old	I.T.	Old
1	3,4-dimethylhex-1-ene	7.56	×														×	×						
2	2-ethylbutanoic acid	8.05													×	×			×		×	×		
3	3,4-dihydroxy-3,4-dimethylhexane-2,5-dione	8.77												×		×								
4	ethyl butyrate	9.26														×								

5	1,1'-bi(cyclohexane)	11.75	×														×	×				
6	glycerol*	11.80							×	×												
7	2-hexyldecan-1-ol	12.30	×																			
8	cyclotetradecane	12.50	×				×	×							×	×					×	×
9	2,3,5,6-tetramethyl-1,4-dioxane-2,5-diol	13.34																		×		
10	2,4-di-*tert*-butylphenol	13.68	×	×			×	×							×	×					×	×
11	hexadecan-1-ol	14.20	×				×	×							×	×					×	×

12	1-(octyloxy)octane	14.74	×																			
13	2,6,10-trimethyltetradecane	15.00	×																			
14	(*E*)-heptadec-15-enal	15.69	×				×	×							×	×					×	×
15	2-methylhexadecan-1-ol	15.92	×																			
16	ethyl pentadecanoate	16.24																×				
17	3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (dextro or levo)*	16.78	×	×	×	×					×	×	×	×					×	×		
18	(*E*)-henicos-10-ene	17.04					×	×														

19	3-isobutylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (dextro or levo)*	17.50			×	×							×	×					×	×				
20	1,3,5-trimethyl-2-octadecylcyclohexane	17.56																						×
21	(*E*)-tetradec-9-enoic acid	17.70		×																				
22	heptadecan-1-ol	18.28	×				×	×							×	×		×			×	×		
23	*N*,*N*-diethyl-10,13-dimethyl-17-(6-methylheptan-2-yl)-4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-amine	18.88																						×

24	3,7,11-trimethyldodecan-1-ol	19.48																			×	×
25	dioctyl adipate	19.59		×							×	×										
26	2,2'-methylenediphenol	19.92								×												
27	bis(2-ethylhexyl) phthalate	20.78	×																			
28	palmitic acid	21.01										×										
29	3-benzylhexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione	21.09																	×	×		



30	6-((*E*)-4-((*E*)-2-(5-hydroxy-2-methylenecyclohexylidene)ethylidene)-7a-methyloctahydro-1H-inden-1-yl)-2-methylheptane-2,3-diol	22.21	×
31	1,2-didodecyldisulfane	25.43	×

*: present in non-inoculated culture media.

These results led us to conclude that more the two bacteria were together, more the mixture will be able to produce volatiles as a response to *Burkholderia* sp.-*B. megaterium* interaction.
All obtained results showed that the long-term storage of tested bacteria influence the secretion of volatiles molecules. Moreover, the response of each bacterium to long-term storage was different, for example, storage for long periods of time decreased the production of volatiles by *Burkholderia* sp., in contrast, the production of volatiles increased by *B. megaterium* and the mixture.

MVOCs after derivatization

To identify other compounds, we have used silylation reaction to generate silylated products with better chromatography proprieties and volatility. Overall, five compounds were identified with no compound detected in cyclohexane extract and no commonly compounds for all three strains. Table 2 showed the list of the different identified silylated compounds. Pentan-2-ol **32** (DCM and BuOH) and glycerol **34** (DCM, EtOAc and BuOH) were the two compounds identified in both *Burkholderia* sp. and mixture of strains. When we compared the detection of glycerol (**6** and **34**) in extract before and after derivatization, we notice that this compound was more detectable after derivatization due to its sensibility. Butan-2,3-diol **33** identified in both immediately treated and stored extracts (DCM, EtOAc and BuOH) from *B. megaterium* and mixture. However, butan-1,2,3-triol **35** was detected only in immediately treated dichloromethane combined strains. Furthermore, 2,3,4-trihydroxybutanal **36**, the only aldehyde compound, was present in both immediately treated and stored *B. megaterium n*-butanol extract. Therefore, except 2,3,4-trihydroxybutanal **36**, never reported before from bacteria of the *Bacillus* gender, all the rest detected compounds were reported for the first time in *Bacillus* and *Burkholderia* sp. but reported from other *Bacillus* and *Burkholderia* species (Kanchiswamy et al., 2015; Uta et al., 2012).
All obtained data led us to confirm that long-term storage of bacteria possessed a slight influence in the production of compounds by *Burkholderia* sp., *B. megaterium* and even by their combined mixture.

Table 2. Compounds identified in different extracts of *Burkholderia* sp., *Bacillus megaterium* and combined strains after silylation.

			***Burkholderia* sp.**						***Bacillus megaterium***						**Mixture**					
			dichloromethane		Ethyl acetate		*n*-Butanol		dichloromethane		Ethyl acetate		*n*-Butanol		dichloromethane		Ethyl acetate		*n*-Butanol	
N°	**Compounds**	**r.t. (min)**	I.T.	Stored	I.T.	Stored	I.T.	Stored	I.T.	Stored	I.T.	Stored	I.T.	Stored	I.T.	Stored	I.T.	Stored	I.T.	Stored
32	pentan-2-ol	11.76	×																×	
33	butan-2,3-diol	12.68							×		×	×	×	×	×	×	×	×	×	×
34	glycerol*	15.82	×		×	×	×	×									×	×	×	

35	butan-1,2,3-triol	16.27			×
36	2,3,4-trihydroxybutanal	16.81	×	×	

*: present in non-inoculated culture media.

Conclusion

Our results showed that the volatile composition, of different rhizobacteria (*Burkholderia* sp., *B. megaterium* and their mixture) extracted with four different solvents (cyclohexane, dichloromethane, ethyl acetate and *n*-butanol) and analyzed by GC-HRMS and derivatization reactions, were mainly composed of alcohols, hydrocarbons, esters and diketopiperazines. . In conclusion, we showed that long-term storage affect microbial volatile composition. Identified chemical classes of compounds motivate us to push the work of test our bacterial extracts and pure compounds in the agronomic fields especially for their potential for plant growth. Further work is needed also to quantify these detected volatile compounds and to improve the impact of long-term storage on the bioactivity of tested bacteria.

References

Bitas, V., Kim, H.S., Bennet, J.W., Kang, S. 2013. Sniffing on Microbes: Diverse Roles of Microbial Volatile Organic Compounds in Plant Health. *Mol Plant Microbe In*. 26, 835-843.

Blom, D., Fabbri, C., Connor, E., Schiestl, F., Klauser, D., Boller, T., Eberl, L., and Weisskopf, L. 2011. Production of plant growth modulating volatiles is widespread among rhizosphere bacteria and strongly depends on culture conditions. *Environ. Microbiol.* 13, 3047-3058.

Huang, Y., Xu, C., K., Ma, L., Zhang, K., Q., Duan, C., Q., Mo, M., H. 2010. Characterisation of volatiles produced from *Bacillus megaterium* YFM3.25 and their nematicidal activity against Meloidogyne incognita. *Eur J Plant Pathol*. 126, 417-422.

Jian-Hua, W., Chun-Shan, Q., Xiao-Hui, Q., Xin, L., Sheng-Di, F., 2010. Determination of diketopiperazines of *Burkholderia cepacia* CF-66 by gas chromatography–mass spectrometry. *Anal. Bioanal. Chem.* 396, 1773–1779.

Kai, M., Crespo, E., Cristescu, S.M., Harren, F.J., Francke, W., and Piechulla, B. 2010. *Serratia odorifera*: analysis of volatile emission and biological impact of volatile compounds on *Arabidopsis thaliana*. *Appl. Microbiol. Biotechnol.* 88, 965-976.

Kai, M., Haustein, M., Molina, F., Petri, A., Scholz, B., Piechulla, B. 2009. Bacterial volatiles and their action potential. *Appl Microbiol Biotechnol* 81:1001-1012.

Kanchiswamy, C., N., Malnoy M., Maffei, M., E. 2015. Chemical diversity of microbial volatiles and their potential for plant growth and productivity. *Front. Plant Sci.* 6, 151.

Ludovic, V., Groleau, M., C., Dekimpe, V., Déziel, E. 2007. *Burkholderia* Diversity and Versatility: An Inventory of the Extracellular Products. J. *Microbiol. Biotechnol.* 17, 1407-1429.

Schulz, S. and Dickschat, J. S. 2007. Bacterial volatiles: the smell of small organisms. *Nat. Prod. Rep*. 24, 814-842.

Uta, E.T, Janine, K., Rene, W., Birgit, P. 2012. Volatile mediated interactions between bacteria and fungi in the soil. *J. Chem. Ecol*. 38:665-703.

Vary, P., S., Biedendieck, R., Fuerch, T., Meinhardt, F., Rohde, M., Deckwer, W., D., Jahn, D. 2007. *Bacillus megaterium* - from simple soil bacterium to industrial protein production host. *Appl Microbiol Biotechnol*. 76, 957-967.

Wang, J., H., Quan, C., S., Qi, X., H., Li, X., Fan. S., D. 2010. Determination of diketopiperazines of *Burkholderia cepacia* CF-66 by gas chromatography–mass spectrometry. *Anal. Bioanal. Chem.* 396, 1773-1779.

Wenclawiak, B. W., et al. 1993. GC-MS-FID Analysis of BSTFA derivatized polar components of diesel particulate matter (NBS SRM 1650) Extract. *Fresenius' J. Anal. Chem.* 6-9, 808-812.

Zou, C., Li, Z., Yu, D. 2010. *Bacillus megaterium* strain XTBG34 promotes plant growth by producing 2-pentylfuran. *J. Microbiol.* 48, 460-466.

Partie B: Impact of long-term storage on the chemical composition and biological activities of *Bacillus megaterium* AGN01 and *Burkholderia* sp. AGN02

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Abstract

In this study, the evolution of chemical composition and biological activities of extracts (cyclohexane, dichloromethane, ethyl acetate and *n*-butanol) from *Burkholderia* sp., *Bacillus megaterium* and their resulting mixture, in function of long-term storage, were investigated. All extracts were evaluated *in vitro* at 50 µg/mL against 5-lipoxygenase, xanthine oxidase and α-amylase enzymes, HCT-116, MCF7 and OVCAR cancer cell lines. The highest phenolics content was obtained in immediately treated *B. megaterium* ethyl acetate extract (39.6±0.8 mg GAE/g DM). All *n*-butanol extracts were able to produce polar aromatic and/or phenolic compounds detected by high performance liquid chromatography (HPLC) at 280 nm. It was found that stored *B. megaterium* ethyl acetate extract possessed the highest anti-inflammatory activity (IP(%)=35.0±2.0%). Moreover, immediately treated *B. megaterium* dichloromethane extract exhibited a very high anti-xanthine oxidase activity (IC_{50}= 7.9±1.4 mg/mL). However, the combined strain showed more powerful anti-diabetic activity (immediately treated dichloromethane extract, IP(%)=70.4±1.2%). Stored *Burkholderia* sp. cyclohexane extract showed the most interesting cytotoxic activity against HCT-116 (IP(%)=64.0±2.7%). Results showed that the long-term storage of bacteria influenced considerably the chemical composition and the biological activities of *B. megaterium* AGN02*, Burkholderia* sp. AGN02 and their combined strains. Therefore, the phenolic amount (polar by HPLC) and toxicity of different bacteria extracts increased under the effect of storage, in contrast, the global extracted quantities from different studied bacteria, have declined when stored.

Keywords

Bacillus megaterium AGN01, *Burkholderia* sp. AGN02, biological activities, HPLC analysis, chemical composition.

Introduction

Interest in microorganisms as a source of pharmacological active compounds has increased worldwide, particularly in the search of new pharmaceutical products including antibiotics, antitumor agents, immunomodulators (Demain and Lancini, 2006). Most of the microorganisms producing bioactive metabolites are bacteria. Most of the products formation are influenced and regulated by the nature of nutrients, growth rate, feedback control, enzyme inactivation, and enzyme induction (Slightom et al., 2009).

B. megaterium, the biggest known bacteria, has been found in a variety of niches, such as soil, plant, water and sediment (Vary, 2007). As it has been demonstrated that *B. megaterium* possesses various biological proprieties as antibiotic, antitumor and nematicide activities and growth promotants of plants (Aksoy and Ozman-Sullivan 2008; Huang et al., 2010). It produces also a large variety of industrial enzymes such as penicillin acylase, several alpha- and beta-amylases, proteases and glucose dehydrogenase (Panbangred et al., 2000; Vary, et al., 2007).

Traditionally, *Burkholderia* species have been known as human, animal and plant pathogens (Ludovic et al., 2007). However, several *Burkholderia* species secrete extracellular bioactive products such as toxins, antibiotics, antitumor agents (Ludovic et al., 2007; De Soyza et al., 2008). They produce also a large variety of enzymes such as proteases, lipases, chitinases and phospholipases C (Ludovic et al., 2007).

However, the impact of long-term storage on the chemical composition and biological activities of *Bacillus megaterium* AGN01, *Burkholderia* sp. AGN02 and their combined mixture have never been studied. This study aims to valorize two different bacteria co-exist in agriculture soil, in order to find new bioactive microbial products. In addition, the influence of long-term storage on the chemical composition and biological activities of different organic extracts obtained from *B. megaterium, Burkholderia* sp. and their mixture have been explored. This study intends to quantify phenolic compounds of various extracts (cyclohexane, dichloromethane, ethyl acetate and *n*-butanol) of *Burkholderia* sp., *B. megaterium* and the resulting combined mixture and to evaluate their potential as anti-inflammatory, anti-xanthine oxidase, anti-acetylcholenesterase, anti-diabetic and cytotoxic agents. Moreover, the influence of long-term storage on the production of bioactive molecules was investigated.

Materials and methods

Chemicals

All chemicals were of analytical reagent grade and purchased from Sigma-Aldrich-Fluka (Saint-Quentin, France).

Identification and culture of studied bacteria

The two studied strains have been isolated from samples of agricultural soils used to grow barley and wheat, by the dilution plating technique and their ability to solubilize tri-calcium phosphate. These two strains have been characterized morphologically and identified by sequencing of the bacterial 16 S rRNA gene after PCR amplification using the universal bacterial primers 27f and 1492r. The resulting sequences were compared to NCBI Nucleotides database by BLAST giving an identification as *Bacillus megaterium* (AGN01) and *Burkholderia* sp. (AGN02). The phylogenetic trees have also been established.

Bacterial strains and culture media were obtained from the culture collection of Agronutrition (Agronutrition, Labège France). *Burkholderia* sp. AGN02 was grown in a modified Bennett broth (water 1000mL; glycerol 6.3 g/L; peptone from soybean 2.5 g/L; yeast extract 1.5 g/L; pH 7.2) at 30°C during 24h under agitation. *B. megaterium* AGN01 was grown in a modified Bennett broth (water 1000mL; dextrose 6.3 g/L; peptone from soybean 2.5 g/L; yeast extract 1.5 g/L; pH 7.2) at 30°C during 24h under agitation. A total volume of 5000 mL of bacterial cultures was obtained and split in two. Half of this bacterial culture was centrifuged at 10^4 g for 20 min at 4°C and filtered through a 0.22 µm filter, to obtain cell free culture filtrate stored at 4°C during seven days before extraction (called immediately treated). The second part of the bacterial culture was stored at 4°C during three months and then filtered through a 0.22 µm filter before extraction.

Extraction

Each filtered supernatant was separately subjected to extraction with different solvents of croissant polarity: cyclohexane, dichloromethane, ethyl acetate and *n*-butanol. All organic extracts were evaporated to dryness by rotary evaporation under vacuum at 30°C.

Total phenolic amount

The phenolics of each strain extract were determined by "Folin-Ciocalteu" reagent assay (Bekir et al., 2013). 20 µL of each extract solution (3 mg/mL) was mixed with 100 µL of Folin-Ciocalteu reagent (0.2 N). The mixture was then agitated for 30 sec and allowed to stand over dark for 5 min and then 80 µL of sodium carbonate solution diluted with distilled water (75 g/L) was added and agitated. After 15 min of incubation, absorbance was measured

at 765 nm. A standard calibration curve was plotted using gallic acid prepared at different concentrations ranging from 0 to 30 mg/L. The result of quantification was expressed as mg of gallic acid equivalent per gram of dry mass (mg GAE/g DM).

HPLC analysis

The HPLC analysis of butanolic extract was performed in an ultimate 3000 pump- Dionex and Thermos Sepration products detectors UV-150 model. Different obtained butanolic extracts were prepared by dissolving 20 mg in 1 mL (90:10 v/v) water/acetonitrile (ACN), then were filtered through a Millex HA 0.25 µm filter. Chromatographic separation was performed, at ambient temperature, on a Phermerex RP-C18 column, 25 cm x 4,6 mm and particle size 5µm, on equipped with a 5 µm C18 guard column. The mobile phases were (A) acidified water (acetic acid, pH = 2.65) and (B) water/ACN (20:80 v/v, pH = 2.65). The gradient used was 0–35 min, 0.1–30% B; 35–40 min, 30–50% B; 40–45 min, 50-99.9% B and 45-60 min, 99.9-0.1% B. 20µL of samples was injected and the detection was performed at 280 nm with a solvent flow rate was 1.2 mL/min.

Anti-5-lipoxygenanse assay

Anti-inflammatory evaluation was assessed by determination of percentage of inhibition of 5-lipoxygenanse (**5-**LOX) using spectrophotometric measurement (Bekir et al., 2013). Various extracts were tested at the concentration of 50 µg/mL in 96-well plates incubated at 25°C for 10 min. Briefly, in a 96-well plate, 150 µL phosphate buffer (pH 7.4) were mixed with 20 µL extract (50 µg/mL), 60 µL of linoleic acid (3.5 mM) and 20 µL of 500 U 5-lipoxidase enzyme solution. After 10 min of incubation over dark at 25°C, the absorbance was measured at 234 nm in a MULTISKAN GO spectrophotometer. The activity was defined as the percentage of inhibition of the 5-lipoxygenase enzyme. The nordihydroguaiaretic acid (NDGA), a potent anti-5-LOX, was used as standard.

Anti-xanthine oxidase activity

The xanthine oxidase inhibition was assessed using the spectrophotometric measurement of formation of uric acid from xanthine at 295 nm (Kammoun El Euch et al., 2015). Briefly, 60 µL of 70 mM sodium phosphate buffer (pH 7.5), 30 µL of 0.1 µM xanthine oxidase enzyme and 50 µL of extract (200 µg/mL) were placed in wells of 96-well plates and mixed for 30 sec. After 15 min pre-incubation over dark at 25°C, 60 µL of xanthine was added. The mixture was further incubated for 10 min and the absorbance was measured at 295 nm. The

positive control was prepared using the same procedure expects the extract was replaced by allopurinol, known as a potent anti-xanthine oxidase.

Anti-α-amylase activity

The α-amylase inhibitory assay was carried out using of a modified procedure described by Shalaby et al., (2014). A 50 µL of α-amylase solution (0.58 mg/mL) was mixed with different extracts at 50 µg/mL. The mixture was pre-incubated and boiled at 25°C for 10 min, after which 100 µL of 1% of starch solution was added to the content of the tube and then was further incubated for 3 min at 25°C. Finally, the reaction was terminated by adding 100 µL of dinitrosalicylic acid reagent and the mixture was incubated in boiling water (100°C) for 10 min. The mixture was diluted with 1 mL sodium phosphate buffer solution (pH 6.9) and the absorbance was measured at 530 nm using MULTISKAN GO spectrophotometer (Thermofisher, France). A positive control was prepared using the same procedure replacing the extract with acarbose. The activity was calculated as a percentage of inhibition of the α-amylase.

Cytotoxic assay

Cytotoxcity of extracts was estimated on human colon cancer (HCT-116), human breast cancer (MCF-7) and human ovarian cancers (OVAR) described by Kammoun El Euch et al., (2015). Different cells were distributed 96-well plates at a concentration of 10^4 cells/well in 100 µL. Plates were pre-incubated for 24 h at 37°C 100 µL of culture medium containing samples at 50 µg/mL was added per well and then further incubated for 48h in a humidified atmosphere with 5% CO_2 at 37°C. Next, the medium was removed and replaced with 50 µL of 1 mg/mL MTT and incubated for a further 40 min at 37°C. The MTT solution was then discarded from all plates and 50 µL of DMSO, used as a solubilizing agent to dissolve the formazan crystal, were added. Finally, the optical density was measured at 605 nm using a MULTISKAN GO spectrophotometer. The cytotoxic effects of different extracts were estimated in terms of cell population growth inhibition percentage. Tamoxifen was used as positive control.

Statistical Analysis

All data were expressed as means ± standard deviations of three triplicate measurements. Differences between the means were established using ANOVA test and standard deviations (S.D.) did not exceed 5% for the majority of the obtained values.

Results and discussion

This is the first study that investigated the impact of long-term storage on the chemical composition (total phenolic content and HPLC), anti-inflammatory, anti-xanthine oxidase, anti-α-amylase and anti-cancer activities of *B. megaterium, Burkholderia* sp. and their mixture extracts.

Extraction yields

A sequential method was adapted to obtain different extracts and presented as follows: cyclohexane, dichloromethane, ethyl acetate and *n*-butanol (Table 1). Result showed that the total quantities from different immediately treated strains (*Burkholderia* sp., *B. megaterium* and their mixture) were higher than those extracted from stored ones indicating that the production of molecules by bacteria, whatever their nature, have declined when stored or or have been consumed or transformed.

Table 1. Extraction quantities and total phenolic content of different extracts obtained by *Burkholderia* sp. *B. megaterium* and their mixture.

Samples			Extracted quantities (mg/L)	Phenolics (mg GAE/gDM)*
Burkholderia sp.	Cyclohexane	I.T.	11	nd
		Stored	15	8.2±0.4[i,j]
	Dichloromethane	I.T.	17	26.9±2.0[d]
		Stored	41	8.2±1.3[i,j]
	Ethyl acetate	I.T.	101	30.4±1.3[c]
		Stored	116	20.2±0.8[f]
	n-Butanol	I.T.	1023	6.3±0[k]
		Stored	732	14.2±0.9[h]
B. megaterium	Cyclohexane	I.T.	25	nd
		Stored	24	nd
	Dichloromethane	I.T.	279	14.9±1.2[g,h]
		Stored	112	8.9±0.8[i,j]
	Ethyl acetate	I.T.	781	39.6±0.8[a]
		Stored	261	24.8±0.5[d,e]
	n-Butanol	I.T.	841	23.0±1.0[e]

Mixture		Stored	589	30.5±3.0^{b,c}
	Cyclohexane	I.T.	13	nd
		Stored	13	nd
	Dichloromethane	I.T.	93	9.8±3.6^{i,j}
		Stored	93	16.3±2.4^{g,h}
	Ethyl acetate	I.T.	334	29.2±4.1^{b,c}
		Stored	417	6.9±0.9^{j,k}
	n-Butanol	I.T.	1115	15.5±2.0^{g,h}
		Stored	684	17.6±2.2^{g,h}

I.T.: immediately treated. nd: not detected. Means with the same letters in a column are not significantly different at $P<0.05$.

Results showed also that the highest quantities from each bacterium were recorded in polar solvents (101-1115 mg/L): *n*-butanol, followed by ethyl acetate; dichloromethane and cyclohexane extracts (11-279 mg/L) were poor. This variation was due to the nature of molecules. A significant modification in the extracted quantities was observed for the *n*-butanol extract from *Burkholderia* sp. and the mixture. Extracted quantities decreased when stored from 1023 to 732 and from 1115 to 684 mg/L for *Burkholderia* sp. and the mixture, respectively. However, a slight modification in the extracted quantities was observed for non-polar (cyclohexane and dichloromethane) and ethyl acetate extracts from *Burkholderia* sp. and the mixture.

For *B. megaterium*, results showed that extracted quantities was highly affected by long term-storage. Expect cyclohexane extract, which contain similar quantities, the rest of immediately treated *Bacillus* extracts have more yields than stored ones. This variation might be due to the nature of molecules.

Total phenolic amount

Results were presented in Table 1 and showed that polar extracts (ethyl acetate and *n*-butanol extracts) have the higher phenolics values. All dichloromethane and ethyl acetate extracts from immediately treated of *B. megaterium* and *Burkholderia* sp. have the higher phenolics amounts, compared to stored ones, in contrary to *n*-butanol extract. In addition, *n*-butanol and dichloromethane extracts from stored mixture have the higher phenolics amounts compared to immediately treated ones. These results indicated that long-term storage influenced the production of phenolics for different studied bacteria.

HPLC analysis

n-Butanol extracts (*Burkholderia* sp., *B. megaterium* and their mixture) were chosen to be analyzed by HPLC, at 280 nm, due to their sufficient quantities. Many other compounds with low intensity (0-200 mV) were detected in all *n*-butanol extracts (between 10 and 50 min). The chromatograms showed that *n*-butanol extracts contained polar compounds (between 2 and 10 min). Overall, eight compounds, with high intensity (> 200 mV) or peak area, were present in different *n*-butanol extracts. Five compounds were commons for all *n*-butanol extracts (**1**, **3**, **4**, **5** and **8**). However, compound **2** was present in immediately treated and stored *n*-butanol extracts from *B. megaterium* and the mixture. Compound **7** was present only in the cultures of *B. megaterium,* mostly in immediately treated extract.

For *B. megaterium*, the compound **2** (retention time 2.08 min) decreased after storage, in contrast, the compounds **3**, **4, 6 and 7** increased after storage. For *Burkholderia* sp., storage has resulted in increase of compounds **1**, **4** and **5**. As regards the mixture of bacteria, decrease in the compound **1** was accompanied by increase of the compounds **2**, **3**, **4**, **6** and **8**.

The quantitative changes of the detected compounds showed that only compound **4** has the same behavior (increase) in all mixtures. All these phenolic compounds increase during storage with the exception of the compounds **2** in *B. megaterium* and **1** in the mixture. It is possible that these decreases and increases are happening between compounds (1-8) following related biotransformation in storage.

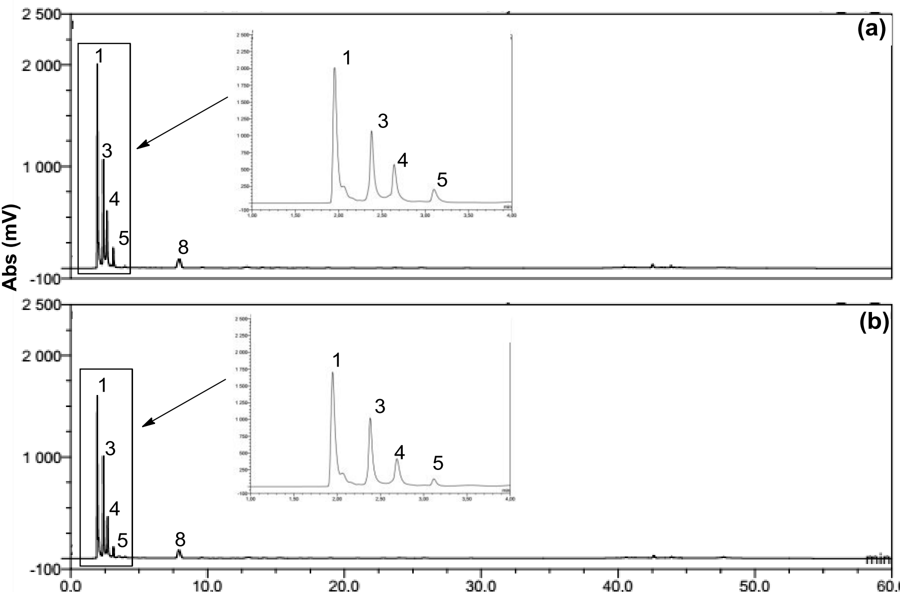

Figure 1. HPLC chromatograms of stored *Burkholderia* sp. butanolic extract (a) and immediately treated *Burkholderia* sp. butanolic extract (b).

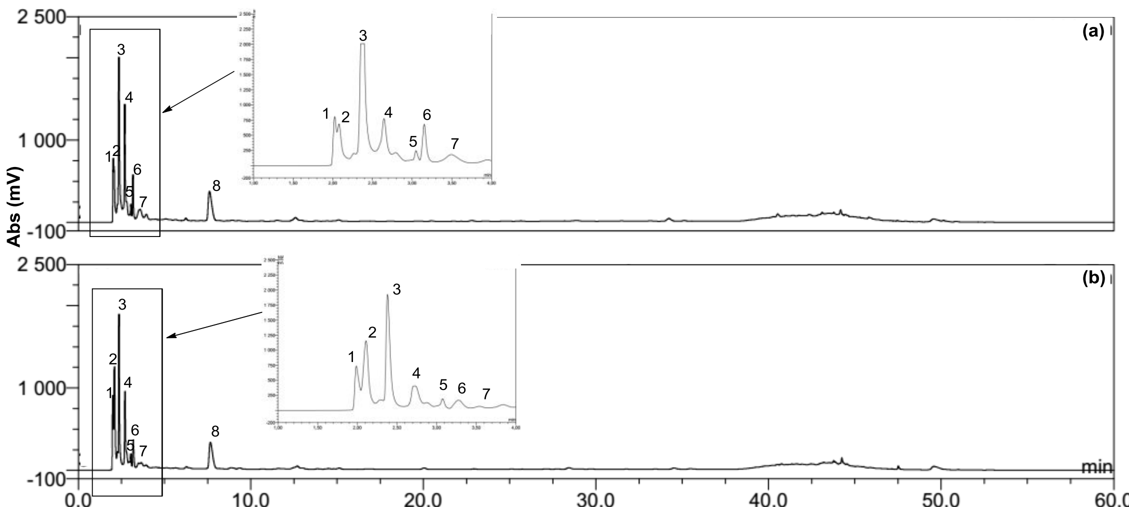

Figure 2. HPLC chromatograms of stored *B. megaterium* butanolic extract (a) and immediately treated *B. megaterium* butanolic extract (b).

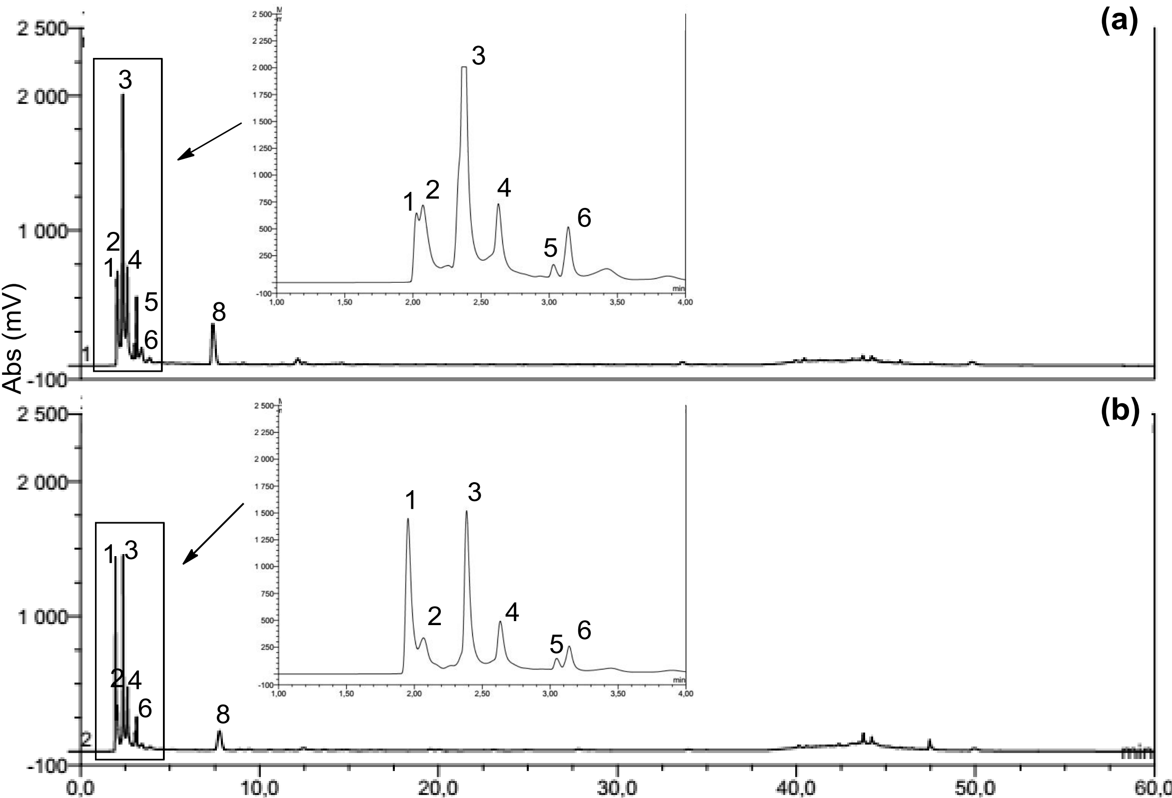

Figure 3. HPLC chromatograms of stored mixture butanolic extract (a) and immediately treated mixture butanolic extract (b).

Anti-inflammatory activity

The effects of different extracts, tested at 50 µg/mL, on the inhibition of the 5-LOX enzyme were summarized in Table 2. All extracts of both strains (*B. megaterium* and *Burkholderia* sp.) inhibited the 5-LOX enzyme with slight to moderate percentage inhibition with the high

IP% value recorded for stored *B. megaterium* ethyl acetate extract (IP%= 37.5±2.0%). We notice that ethyl acetate extracts from *B. megaterium* and *Burkholderia* sp. exhibited the highest anti-5-LOX activity. In addition, mixture extracts exhibited a very slight anti-5-LOX activity with value ranged from 2.8±1.6 to 18.7±2.5%. We should notice that a slight influence was seen after storage the inhibition of 5-LOX enzyme with the higher IP% values recorded for stored strains in almost cases. In conclusion, no great significant influence of long-term storage on the inhibition of 5-LOX enzyme was observed.

Anti xanthine oxidase

The anti-xanthine oxidase activity of *Burkholderia* sp., *B. megaterium* and their mixture extracts was tested at 50 µg/mL (Table 2). All extracts of immediately treated *Burkholderia* sp. inhibited the xanthine oxidase enzyme with slightly percentage (4.5±1.3 to 30.5±0.7%), but for stored ones, extracts inhibited significantly (14.7±1.2--67.4±0.8%).

For *B. megaterium*, except cyclohexane extract which showed a great difference in IP% values in function of long term storage with an IP% recorded for stored one (48.8±0.2%) was ten time higher than value recorded with immediately treated one (4.3±1.6%), all extracts showed similar anti-xanthine oxidase activity with a significant activity registered for both dichloromethane extracts (81.5±0.7 and 80.2±1.1% for immediately treated and stored ones, respectively). The anti-xanthine oxidase activity of these two samples was expressed by IC50. Therefore, the immediately treated dichloromethane extract has three time stronger anti-xanthine oxidase activity (IC50=7.9±1.4 mg/mL) when compared to the same extract after storage (IC50=24.2±1.3 mg/mL). So, the fractionation of dichloromethane extract is important to identify pure active compounds against xanthine oxidase enzyme, more active than allopurinol (IC50=1.13 ±0.78 mg/L).

When *B. megaterium* was combined with *Burkholderia* sp., the anti-xanthine oxidase activity of cyclohexane, ethyl acetate and *n*-butanol extracts increased with time of storage, contrary to dichloromethane which exhibited similar powerful activity (67.5±1.2 and 65.7±1.5%). We can conclude that long-term storage affected the production of molecules exhibiting anti-xanthine oxidase potential.

Table 2. Anti-inflammatory (5-lipoxygenase), antidiabetic (α-amylase) and anti-xanthine oxidase activities of *Burkholderia* sp., *B. megaterium* and mixture extracts (50 µg/mL).

Extract			Anti-inflammatory activity (%)	Anti-xanthine oxidase activity (%)	anti-α-amylase activity (%)
Burkholderia sp.	Cyclohexane	I.T.	11.6±1.6[i]	4.5±1.3[i]	42.3±1.0[g,h]
		Stored	18.7±1.3[g]	14.7±1.2[g]	61.5±0.4[b,c]
	Dichloromethane	I.T.	15.3±0.5[g,h]	16.4±2.1[g]	44.0±1.2[f,g,h]
		Stored	7.7±3.2[k]	67.4±0.8[b]	69.8±1.3[a]
	Ethyl acetate	I.T.	23.1±2.3[f]	30.5±0.7[f]	62.5±1.8[a,b,c]
		Stored	25.3±2.3[e,f]	39.9±1.8[e]	59.6±1.9[c,d]
	n-Butanol	I.T.	9.1±1.9[j,k]	7.8±3.1[h,i]	11.1±0.5[k]
		Stored	8.0±2.1[j,k]	34.6±4.2[f]	49.6±1.8[e,f]
B. megaterium	Cyclohexane	I.T.	8.7±1.9[j,k]	4.3±1.6[i]	69.3±1.0[a]
		Stored	28.9±1.8[d,e]	48.8±0.2[c]	63.4±0.6[a,b,c]
	Dichloromethane	I.T.	15.2±3.6[g,h]	81.5±0.7[a] (IC_{50}=7.9±1.4)*	37.8±1.7[h]
		Stored	13.8±2.7[h,i]	80.2±1.1[a] (IC_{50}=24.2±1.3)*	69.3±1.4[a]
	Ethyl acetate	I.T.	30.7±0.2[c,d]	33.2±2.1[f]	67.8±1.1[a,b]
		Stored	37.5±2.0[b]	31.9±3.8[f]	58.1±0.6[c,d,e]
	n-Butanol	I.T.	9.7±1.7[j,k]	6.6±2.9[h,i]	22.7±0.4[j]
		Stored	13.6±3.4[h,i]	7.1±3.8[h,i]	26.9±0.7[i,j]
Mixture	Cyclohexane	I.T.	3.7±1.7[k,l]	na	51.9±0.6[d,e,f]

	Stored	11.4±1.3^{i,j}	15.1±0.8^{g}	7.8±0.1^{k}
Dichloromethane	I.T.	na	67.5±1.2^{b}	70.2±1.2^{a}
	Stored	6.9±2.5^{k}	65.7±1.5^{b}	36.9±0.2^{h,i}
Ethyl acetate	I.T.	18.7±2.5^{g,h}	16.0±1.3^{g}	22.9±0.3^{j}
	Stored	2.8±1.6^{l}	41.1±1.0^{d,e}	43.7±2.5^{f,g,h}
n-Butanol	I.T.	na	na	38.0±0.7^{h}
	Stored	4.1±1.5^{k,l}	13.2±2.6^{g,h}	28.3±0.7^{i,j}
NDGA (2.0 µg/mL)		53.1±0.1^{a}	-	-
Allopurinol (1.0 µg/mL)		-	46.2±0.2^{c}	-
Acarbose (50 µg/mL)		-	-	48.4±0.9^{f,g}

*: mg/L. I.T.: immediately treated. NDGA: nordihydroguaiaretic acid. na: not active. Data are the means of three independent experiments±standard deviations (n=3). Means with the same letters in a column are not significantly different at $P<0.05$.

Anti-α-amylase activity

Anti-diabetic activity of *Burkholderia* sp., *B. megaterium* and their mixture extracts was tested (Table 2) at a concentration of 50 µg/mL using α-amylase enzyme. We notice the presence of an interesting anti-diabetic activity. From Table 2, we observed that stored *Burkholderia* sp. extracts exhibited more interesting inhibitory activity against the enzyme α-amylase (IP(%) ranged from 49.6±1.8 to 69.8±1.3%) in comparison to immediately treated extracts (IP(%) ranged from 11.1±0.5 to 62.5±1.8%). For *B. megaterium*, both immediately treated and stored extracts exhibited good anti-diabetic activity. The best activity was observed in immediately treated cyclohexane extract (IP(%)= 69.3±1.0%) and stored dichloromethane extract (69.3±1.4%).

In the other hand, Table 2 showed that combined strains extracts exhibited moderate anti-diabetic activity but a good activity was observed in immediately treated dichloromethane extract (IP(%)= 70.4±1.2%). These results proved the presence in some extracts interesting and more potent anti-α-amylase compared to acarbose.

Cytotoxic activity

Cytotoxicity evaluation against three cell lines (OVCAR, MCF-7 and HCT-116) were demonstrated at 50 µg/mL (Table 3). By comparing the IP(%),various extracts showed that the OVCAR line is the most sensitive to the various extracts . Stored *Burkholderia* sp. extracts exhibited a good cytotoxic activity against all cell lines; IP(%) were from 29.9±5.1 to 64.0±2.7, 15.2±1.3 to 31.0±2.1 and 40.8±1.1 to 50.7±2.5% against HCT-116, MCF-7 and OVCAR cell lines, respectively). On the other hand, the immediately treated *Burkholderia* sp. extracts exhibited moderate cytotoxic activity against all cell lines with IP(%) values varied from 4.7±0.5 to 22.5±2.0% against HCT-116 cell line, from 27.6±3.0 to 41.4±4.2% against OVCAR cell line and from 0 to 30.4±2.2% o against MCF-7 line). These results proved the great influence of storage on the production of toxic molecules by *Burkholderia* sp. strain.

For *B. megaterium*, both stored and immediately treated extracts showed moderate activity against all cell lines. IP(%) values were ranged from 3.0±1.4 to 24.1±5.8%, from 4.0±0.8 to 11.1±0.9 and from 15.0±0.8 to 30.8±1.0% against HCT-116, MCF-7 and OVCAR cell lines, respectively.

In contrast, when *B. megaterium* was combined with *Burkholderia* sp., results showed that the storage of tested strain strongly affects the cytotoxic activities. The anticancer activity of stored mixture extracts were moderate (IP(%)=15.7±1.5-59.1±4.6%, 8.8±1.7-22.1±1.7% and 41.3±7.7-52.1±2.2% against HCT-116, MCF-7 and OVCAR cell lines, respectively) but more

interesting than immediately treated extracts found to possess slight activity (IP(%)=4.3±1.7-12.2±3.6%, 2.2±0.6-2.9±0.7% and 20.7±3.2-34.8±3.4% against HCT-116, MCF-7 and OVCAR cell lines, respectively).
These results indicate that the effect of long-term storage depends on the nature of extracted molecules and the nature of tested bacterium.

Table 3. Cytotoxic activity of different extracts obtained by *Burkholderia* sp., *B. megaterium* and the mixture (tested at 50 µg/mL).

Extract			**Anticancer activity** % inhibition		
			HCT116	MCF7	OVCAR
Burkholderia sp.	Cyclohexane	I.T.	22.5±2.0^{h}	30.4±2.2^{b}	40.3±1.8^{f,g}
		Stored	64.0±2.7^{a}	31.0±2.1^{b}	40.8±1.1^{g}
	Dichloromethane	I.T.	17.4±3.5^{i}	13.4±2.0^{g}	41.4±4.2^{f}
		Stored	29.9±5.1^{f}	24.6±1.3^{c}	42.6±2.2^{e}
	Ethyl acetate	I.T.	na	na	27.6±3.0^{k,l}
		Stored	38.6±3.8^{d}	15.2±1.3^{f}	46.0±0.9^{d}
	n-Butanol	I.T.	4.7±0.5^{o}	na	29.6±4.2^{j}
		Stored	31.3±2.4^{e}	25.1±1.9^{c}	50.7±2.5^{c}
B. megaterium	Cyclohexane	I.T.	16.8±0.2^{j}	4.0±0.8^{l}	30.8±1.0^{i}
		Stored	24.1±5.8^{g}	7.9±1.3^{j}	24.8±1.4^{m}
	Dichloromethane	I.T.	12.6±2.2^{l}	na	27.1±2.0^{l}
		Stored	12.8±1.9^{l}	na	16.2±1.8^{p}
	Ethyl acetate	I.T.	3.0±1.4	11.1±0.9^{h}	18.7±1.4^{o}
		Stored	8.4±2.7^{m}	6.2±0.4^{k}	28.6±4.0^{j,k}
	n-Butanol	I.T.	7.1±2.9^{n}	9.5±1.1^{i}	15.0±0.8^{q}
		Stored	na	8.8±1.2^{i,j}	29.8±0.4^{i,j}
Mixture	Cyclohexane	I.T.	12.2±3.6^{l}	2.2±0.6	24.6±2.7^{m}
		Stored	51.0±1.7^{c}	22.1±1.7^{d}	52.1±2.2^{b}
	Dichloromethane	I.T.	6.2±1.1^{n,o}	na	34.5±4.8^{h}
		Stored	59.1±4.6^{b}	8.8±1.7^{i,j}	51.4±4.2^{b,c}
	Ethyl acetate	I.T.	5.7±2.2^{o}	na	34.8±3.4^{h}
		Stored	21.6±1.1^{h}	17.0±1.7^{e}	41.3±7.7^{f,g}
	n-Butanol	I.T.	4.3±1.7^{p}	2.9±0.7^{m}	20.7±3.2^{n}

	Stored	15.7±1.5[k]	11.2±2.1[h]	41.9±4.1[e,f]
Tamoxifen (0.2 µg/mL)		48.6±2.3[c]	47.2±4.3[a]	61.8±4.2[a]

I.T.: treated immediately. na: not active. Data are the means of three independent experiments±standard deviations (n=3). Means with the same letters in a column are not significantly different at $P<0.05$.

Conclusion

The present study highlighted the significant influence of long-term storage in the chemical composition of the secondary metabolites of *B. megaterium*, *Burkholderia* sp. and their resulting mixture as well as its significant influence on the biological activities. The concentrations of phenolic compounds were determined to be highest in *B. megaterium* extracts. All tested strains were able to produce polar aromatic and/or phenolic compounds that were detected by HPLC coupled with UV detector set at 280 nm. Almost all dichloromethane extracts are endowed with very good anti-xanthine oxidase and anti-diabetic activities. Furthermore, a good cytotoxic activity, especially against OVCAR and HCT-116 cell lines, were recorded for extracts. Therefore, the most toxic extracts were from stored *Burkholderia* sp. (cyclohexane IP(%)=64.0±2.7%) and from stored mixture (dichloromethane IP(%)=59.1±4.6%) against HCT116 cell line. Most of results allowed to show without any doubt the influence of the growth rate of bacteria on the production of specific bioactive molecules. Therefore, the extracted quantities from different studied bacteria, whatever their nature, have declined when stored, in contrast, phenolics amount (polar by HPLC) increased under the effect of storage (biotransformation or defense of the bacteria) and the production of toxic molecules against OVCAR and MCF7 cell lines increased after storage. Further studies are in progress to push the work to identify specific interesting molecules responsible for observed biological activities by bio-guided fractionation.

References

Aksoy, H., M., and Ozman-Sullivan, S., K. 2008. Isolation of *Bacillus megaterium* from *Aphis pomi* (homoptera: aphididae) and assessment of its pathogenicity. Journal of Plant Pathology. 90, 449-452.

Bekir, J., Mars, M., Souchard, J.P., Bouajila, J., 2013. Assessment of antioxidant, anti-inflammatory, anti-cholinesterase and cytotoxic activities of pomegranate (*Punica granatum*) leaves. *Food. Chem. Toxicol.* 55, 470-475.

Slightom, R.N., Buchan, A. 2009. Surface Colonization by Marine Roseobacters: Integrating Genotype and Phenotype . *App. Env. Microbiol.* 75, 6027-6037.De Soyza, A., Silipo, A., Lanzetta, R., Govan, J., R., Molinaro, A. 2008. Chemical and biological features of *Burkholderia cepacia* complex lipopolysaccharides. *Innate Immunity* 14, 127

Demain, A., L., and Lancini, G. 2006. Bacterial Pharmaceutical Products. The Prokaryotes. A handbook on the biology of bacteria. Dowrkin, M., Falkow, S., Rosenberd, E., Schleifer, K., Stackebrandt, E. *3rd ed. Springer, New York, USA*. 1 , 812-833.

Huang, Y., Xu, C., K., Ma, L., Zhang, K., Q., Duan, C., Q., Mo, M., H. 2010. Characterisation of volatiles produced from *Bacillus megaterium* YFM3.25 and their nematicidal activity against *Meloidogyne incognita*. *Eur J Plant Pathol.* 126, 417-422.

Kammoun El Euch, S., Bouajila J., Bouzouita N., 2015. Chemical composition, biological and cytotoxic activities of *Cistus salviifolius* flower buds and leaves extracts. *Ind. Crops Prod.* 76, 1100-1105.

Ludovic, V., Groleau, M., C., Dekimpe, V., Déziel, E. 2007. *Burkholderia* diversity and versatility: An inventory of the extracellular products. *J. Microbiol. Biotechnol.* 17,1407-1429.

Shalaby, N., M., M., Abd-Alla, H., I., Aly, H. F., Albalawy, M. A., Shaker, K. H., Bouajila, J.,2014. Preliminary *in vitro* and *in vivo* evaluation of antidiabetic activity of *Ducrosiaanethifolia* Boiss and its linear furanocoumarins. *BioMed. Res. Int.* ID 480545, 1-13.

Vary, P., S., Biedendieck, R., Fuerch, T., Meinhardt, F., Rohde, M., Deckwer, W., D., Jahn, D. 2007. *Bacillus megaterium*-from simple soil bacterium to industrial protein production host. *Appl Microbiol Biotechnol.* 76, 957-967.

Conclusion

Dans ce présent chapitre de thèse, nous avons développé plusieurs études dans le but d'étudier l'impact du stockage des bactéries sur la composition chimique (dosages des phénoliques, analyse par HPLC et analyse des composés volatils par GC-HRMS) de *B. megaterium* AGN01, *Burkholderia* sp.AGN02 et leur mélange, ainsi que son impact sur les activités biologiques. Le bilan de différents points abordés au cours de cette partie de thèse peut être résumé comme suit :

- Nous avons mis en évidence, dans un premier lieu, l'influence notable du stockage sur la composition chimique des métabolites secondaires et sur les activités biologiques de *B. megaterium*, *Burkholderia* sp. et leur mélange résultant. les quantités les plus élevée en phénols ont été enregistrés dans les extraits de *B. megaterium.* Toutes les souches testées étaient capables de produire des composés aromatiques et /ou phénoliques polaires qui ont été détectés par HPLC couplée à un détecteur UV (280 nm). Presque tous les extraits de dichlorométhane sont dotés d'une très bonne activité anti-xanthine oxydase et anti-diabétiques. En outre, une activité cytotoxique significative, en particulier contre les lignées cellulaires OVCAR et HCT-116, ont été enregistrées pour les différents extraits.
- Nos résultats montrent que la composition des composés volatils émis par les différentes rhizobactéries (*Burkholderia* sp., *B. megaterium* et leur mélange), analysée par GC-MS, étaient principalement constitués d'alcools, hydrocarbures, esters et dicétopipérazines. Nous avons montré aussi que le stockage affecte la composition chimique des volatiles microbiens. L'investigation par GC-MS nous a permis d'identifier plus que trente composés volatils dont 1,3,5-trimethyl-2-octadecylcyclohexane, *N*,*N*-diethyl-10,13-dimethyl-17-(6-methylheptan-2-yl)-4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-amine et 6-((*E*)-4-((*E*)-2-(5-hydroxy-2-methylenecyclohexylidene)ethylidene)-7a-methyl-octahydro-1*H*-inden-1-yl)-2-methylheptane-2,3-diol ont été identifiés pour la première fois à partir des bactéries.

Chapitre V : Synthèse d'analogues structuraux de DKP

Introduction

Les dicétopipérazines, des dérivées de pipérazine, forment une très large gamme de composés naturels issus des microorganismes, ce sont les plus petits peptides cycliques connus, couramment issus de la fusion de deux unités d'acide aminées. Ils possèdent une structure commune de type 2,5-dioxopiperazine qui peut être substitué en position 1, 3, 4 et 6.

Les dérivés de dicétopipérazine constituent une nouvelle classe de métabolites secondaires qui sont produits essentiellement par des bactéries, des champignons, des levures et des organismes marins tels que les éponges.

Ces petits peptides cycliques possèdent des structures qui les permettent non seulement de posséder une large gamme d'activités biologiques, mais aussi d'élaborer et de développer d'autres dérivés qui peuvent être plus actifs. Dans la présente partie du travail, nous nous sommes intéressés à la synthèse des nouveaux analogues protégés de DKP en exploitant la mobilité du proton acide du cycle de dicétopiperazine qui pourrait apporter des activités biologiques intéressantes au composé de départ. En plus, tous les analogues synthétisés ont été évalué pour leurs activités biologiques (les mêmes que celles testées pour les extraits des bactéries). Cette partie de thèse fait l'objet d'une publication qui est actuellemnt soumise dans le journal « *Medicinal Chemistry* ».

Synthesis of new arylidene 2,5-diketopiperazines and evaluation of their anti-acetylcholinesterase, anti-xanthine oxidase, anti-diabetic and cytotoxic activities

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Abstract:

BACKGROUND: 2,5-diketopiperazine derivatives are considered to be important classes of cyclic peptides due to their wide range of biological activities.

METHODS: A series of novel soluble mono-protected arylidene 2,5-diketopiperazine derivatives 3a-p has been prepared by application of Claisen-Schmidt condensation of the N,N-diacetyl-diketopiperazine 1 with a series of arylaldehydes. The compounds were characterized by 1D and 2D 1H/13C NMR and ESI-HRMS spectral data and screened in vitro against acetylcholenesterase, xanthine oxidase, α-amylase and cytotoxic (HCT-116, MCF-7 and OVCAR-3) activities.

RESULTS: The greatest activity against α-amylase enzyme (PI=57.8±1.9%) was obtained for compound 3f bearing a phenoxy moiety. Moreover, the results demonstrated that most newly prepared arylidene 2,5-diketopiperazines 3a-p have a moderate to good cytotoxic activity against the three cell lines used. The compound 3g (4-PhCH2O.Ph) was found to be the most cytotoxic against HCT-116, MCF-7 and OVCAR-3 cell lines (PI=83.2±2.4, 89.6±4.9 and 74.4±5.2%, respectively) followed by 3m (2-Br-5-F.Ph) then 3j (4-C2H5-3-NO2.Ph) both exhibited good cytotoxicity against OVCAR-3 (PI=77.0±2.1 and 71.4±0.9%, respectively).

CONCLUSION: The derivative 3g (4-PhCH2O.Ph) was found to be the most cytotoxic against HCT-116, MCF-7 and OVCAR-3 cell lines followed by 3m (2-Br-5-F.Ph) then 3j (4-C2H5-3-NO2.Ph) both exhibited good cytotoxicity against OVCAR-3.

Keywords: synthesis, 2,5-Diketopiperazines, Claisen-Schmidt reaction, biological activities, anti-diabetic activity, cytotoxic activity.

1. INTRODUCTION

Piperazines are a group of naturally occurring organic compounds with a heterocyclic nitrogenous ring. Piperazines have been known for more than a century, while only 2,5-diketopiperazines have attracted attention due to their wide spectrum of their biological properties [1].

The 2,5-diketoiperazines are a group of piperazine with the smallest cyclic peptides ever known [2]. Plants, marine creatures, insects, bacteria, fungi as well as mammalians have been the main source of these compounds.

In addition, it was proved that 2,5-diketopiperazines are related to the inhibition of plasminogen activator inhibitor-1 (PAI-1) [3], various studies have also reported 2,5-diketopiperazines with anti-tumor [4], antiviral (HIV-1) [5], anti-hyperglycaemic [1], antifungal [6] and antibacterial [7] activities.

2,5-diketopiperazines are extensively obtained by extraction from natural sources with a huge variety of biological activities [8], while most have complicated chemical structures. Therefore, many 2,5-diketopiperazines derivatives may be easily synthesized and showed good biological activities [8].

2,5-diketopiperazine derivatives are an important class of cyclic peptide, displaying a wide variety of biological proprieties. However, solubility is one major problem for some active synthetic or natural 2,5-diketopiperazines. Some recent studies have shown that especially non-protected 2,5-diketopiperazine derivatives showed a week solubility which limits their broad use as pharmaceutical therapeutic [9,10]. Perhaps, this problem due to the intermolecular hydrogen bonds and л-л stacking interactions, therefore, a negative influence on their solubility was observed. A useful solution is to replace one or two of the 2,5-diketopiperazine amide hydrogen atoms by introducing protective groups which may interrupt the formation of hydrogen bonding and the л-л stacking interactions resulting in a non-planar structure [11]. Therefore, in the present study, a novel series of protected 2,5-diketopiperazine derivatives were synthesized via the condensation of 2,4-diacetyl-2,5-diketopiperazine with various arylaldehydes, and their anti-acetylcholinesterase, anti-xanthine oxidase and anti-diabetic as well as their cytotoxic activities were evaluated and discussed.

2. Material and methods

Melting points (m.p.) were determined by using capillary tubes (Buchi 510 apparatus). NMR spectra were recorded with Bruker Avance AM-300 spectrometers operating at 300 MHz for 1H, and at 75 MHz for ^{13}C by using $CDCl_3$ as solvent and none deuterated residual solvent as

internal standard. Chemicals shifts (δ) are given in parts per million (ppm) and coupling constants (J) in Hertz. IR spectra were recorded on a Shimadzu IR Affinity-1 fourier transform infrared spectrometer. Shimadzu HRMS-TOF spectrometer was used in the ESI^+ experiment.

2.1. Synthesis

To 1,4-diacetylpiperazine-2,5-dione **1** (1.5 equiv.) in anhydrous DMF, the arylaldehydes **2** (1 equiv.) in presence of Cs_2CO_3 (1.5 equiv.) was added. The mixture was stirred at room temperature for about 3 h then poured into ice-cold water. The solid was then filtered, washed with cooled water for three times and dried using a rotary evaporator. The resulting solid was purified by precipitation on a mixture (Petroleum ether/EtOAc) to give corresponding diketopiperazines **3a-p**.

2.1.1. Compound 3a: 1-acetyl-3-(4-methoxybenzylidene)piperazine-2,5-dione

Yields: 55%, m.p: 194°C, ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 7.90 (1H, s, NH), 7.38 (2H, d, *J* = 8.8 Hz, H-2’, H-6’), 7.13 (1H, s, =CH), 6.99 (2H, d, *J* = 8.8 Hz, H-3’, H-5’), 4.50 (2H, s, H-6), 3.85 (3H, s, CH_3-O), 2.64 (3H, s, H-10). ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 172.50 (C-8), 162.87 (C-2), 160.41 (C-5), 160.32 (C-4’) 130.31 (C-2’, C6’), 124.8 (C-3’, C5’), 124.09 (C-1’), 120.32 (C-3), 115.01 (C-7), 55.44 (CH_3-O), 46.08 (C-6), 27.15 (C-10). FT-IR (neat): 3210, 1680, 1621, 1510, 1470, 1401, 1272 cm^{-1}. HRMS (ESI) (*m/z*) $[M+H]^+$ for ($C_{14}H_{14}N_2O_4$) calculated 275.1030 found 275.1030.

2.1.2. Compound 3b: 1-acetyl-3-(4-(methylthio)benzylidene)piperazine-2,5-dione

Yields: 60%, m.p: 176°C, ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 7.93 (1H, s, NH), 7.34 (2H, d, *J* = 8.5 Hz, H-3’, H-5’), 7.30 (2H, d, *J* = 8.5 Hz, H-2’, H-6’), 7.11 (1H, s, =CH), 4.50 (2H, s, H-6), 2.64 (3H, s, H-10), 2.51 (3H, s, CH_3-S). ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 172.44 (C-8), 162.74 (C-2), 160.06 (C-5), 141.26 (C-4’), 128.97 (C-1’), 128.70 (C-2’, C-6’), 126.55 (C-3’, C-5’), 125.01 (C-3), 119.66 (C-7), 46.04 (C-6), 27.16 (C-10), 15.13 (CH_3-S). FT-IR (neat): 3212, 1700, 1621, 1376, 1270 cm^{-1}. HRMS (ESI) (*m/z*) $[M+H]^+$ for ($C_{14}H_{15}N_2O_3H$) calculated 291.0803 found 291.0799.

2.1.3. Compound 3c: 1-acetyl-3-(4-chlorobenzylidene)piperazine-2,5-dione

Yields: 61%, m.p: 164°C, ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 7.88 (1H, s, NH), 7.45 (2H, d, *J* = 8.0 Hz, H-2’, H-6’), 7.35 (2H, d, *J* = 8.0 Hz, H-3’, H5’), 7.11 (1H, s, =CH), 4.51 (2H, s, H-6), 2.65 (3H, s, H-10-). ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 172.38 (C-8), 162.66 (C-2), 159.72 (C-5), 135.38 (C-4’), 130.87 (C-1’), 129.84 (C-2’, C-6’), 129.82 (C-3’, C-5’), 126.02 (C-3-), 118.45 (C-7), 46.07 (C-6), 27.21 (C-10). FT-IR (neat): 3201, 1707, 1671, 1479, 1413,

1286 cm^{-1}. HRMS (ESI) (*m/z*) $[M+H]^+$ for ($C_{13}H_{12}N_2O_3Cl$) calculated 279.0536 found 279.0544.

2.1.4. Compound 3d: 1-acetyl-3-(4-(trifluoromethyl)benzylidene)piperazine-2,5-dione

Yields: 27%, m.p: 156°C, ^1H-NMR (300 MHz, $CDCl_3$) δ ppm: 8.01 (1H, s, NH), 7.73 (2H, d, *J* = 8.1 Hz, H-1', H-6'), 7.52 (2H, d, *J* = 8.1 Hz, H-3', H-5'), 7.16 (1H, s, =CH), 4.50 (2H, s, H-6), 2.65 (3H, s, H-10-). ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 172.34 (C-8), 162.75 (C-2), 159.49 (C-5), 136.09 (C-1'), 130.83 (q, J_{FC} = 33 Hz, C-4'), 128.88 (C-3), 127.11 (C-2', C-6'), 126.44 (q, J_{FC} = 3.7 Hz, C-3', C-5'), 123.62 (q, J_{FC} = 270.7 Hz, $-CF_3$), 117.75 (C-7), 46.06 (C-6), 27.21 (C-10). FT-IR (neat): 3197, 1710, 1664, 1411, 1370 cm^{-1}. HRMS (ESI) (*m/z*) $[M+H]^+$ for ($C_{14}H_{12}N_2O_3F$) calculated 313.0800 found 313.0800.

2.1.5. Compound 3e: 1-acetyl-3-(4-morpholinobenzylidene)piperazine-2,5-dione

Yields: 46%, m.p: 200°C, ^1H-NMR (300 MHz, $CDCl_3$) δ ppm: 7.92 (1H, s, NH), 7.37 (2H, d, *J* = 8.7 Hz, H-2', H6'), 7.11 (1H, s, =CH), 6.98 (2H, d, *J* = 8.7 Hz, H-3', H-5'), 4.50 (2H, s, H-6), 3.90 (4H, t, *J* = 4.9 Hz, CH_2), 3.27 (4H, t, *J* = 4.9 Hz, CH_2) 2.63 (3H, s, H-10). ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 172.37 (C-8), 162.77 (C-2), 160.34 (C-5), 150.72 (C-4'), 130.17 (C-2', C-6'), 130.17 (C3', C-5'), 123.42 (C-1'), 120.40 (C-3), 115.60 (C-7), 66.24 (C-2'', C-6''), 48.36 (C-3'', C-5''), 45.89 (C-6), 26.99 (C-10). FT-IR (neat): 3287, 2994, 2874, 1708, 1617, 1413, 1302, 1210, 1184 cm^{-1}. HRMS (ESI) (*m/z*) $[M+H]^+$ for ($C_{17}H_{20}N_3O_4$) calculated 330.1454 found 330.1454.

2.1.6. Compound 3f: 1-acetyl-3-(4-phenoxybenzylidene)piperazine-2,5-dione

Yields: 84%, m.p: 180°C, ^1H-NMR (300 MHz, $CDCl_3$) δ ppm: 7.90 (1H, s, NH), 7.42 (2H, d, *J* = 8.2 Hz, H-2', H-6'), 7.39 (2H, dd, J = 8.5, 7.5 Hz, H-3'', H-5''), 7.21 (1H, t, *J* = 7.5, H-4'') . 7.14 (1H, s, =CH), 7.07 (2H, d, *J* = 8.2 Hz, H-3', H-5'), 7.07 (2H, d, *J* = 8.5 Hz, H-2'', H-6''), 4.51 (2H, s, H-6), 2.65 (3H, s, H-10). ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 172.46 (C-8), 162.73 (C-2), 160.08 (C-5), 158.64 (C-1''), 155.83 (C-4'), 130.29 (C-2', C-6'), 130.01 (C-3'', C-5''), 126.84 (C-1'), 124.86 (C-4''), 124.35 (C-3), 119.75 (C-3', C-5'), 119.64 (C-7), 118.97 (C-2'', C-6''), 46.07 (C-6), 27.19 (C-10). FT-IR (neat): 3207, 1702, 1617, 1386 cm^{-1}. HRMS (ESI) (*m/z*) $[M+H]^+$ for ($C_{19}H_{17}N_2O_4$) calculated 337.1188 found 337.1182.

2.1.7. Compound 3g: 1-acetyl-3-(4-(benzyloxy)benzylidene)piperazine-2,5-dione

Yields: 50%, m.p: 166°C, ^1H-NMR (300 MHz, $CDCl_3$) δ ppm: 7.93 (1H, s, NH), 7.44 (2H, d, *J* = 8.7 Hz, H-2', H-6'), 7.40 (5H, m, Ar-H). 7.12 (1H, s, =CH), 7.05 (2H, d, *J* = 8.7 Hz, H-3', H-5'), 5.11 (2H, s, CH_2-O), 4.50 (2H, s, H-), 2.64 (3H, s, H-10). ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 172.47 (C-8), 162.81 (C-2), 160.29 (C-5), 159.52 (C-4'), 136.20 (C-1''), 130.31 (C-2', H-6'), 128.69 (C-3'', C-5''), 128.22 (C-4''), 127.41 (C-2'', C-6''), 125.05 (C-1'), 124.16 (C-3),

120.16 (C-7), 115.89 (C-3', C-5'), 70.13 (CH_2-O), 46.03 (C-6), 27.14 (C-10). FT-IR (neat): 3176, 1714, 1627, 1405, 1289 cm^{-1}. HRMS (ESI) (*m/z*) $[M+H]^+$ for ($C_{20}H_{19}N_2O_4$) calculated 351.1345 found 351.1347.

2.1.8. Compound 3h: 1-acetyl-3-(4-(1,1,2,2-tetrafluoroethoxy)benzylidene)piperazine-2,5-dione

Yields: 44%, m.p: 174°C, ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 7.88 (1H, s, NH), 7.43 (2H, d, *J* = 8.4 Hz, H-2', H-6'), 7.32 (2H, d, *J* = 8.4 Hz, H-3', H-5'), 7.14 (1H, s, =CH), 6.12 (1H, tt, *J* = 52.8, 2.4 Hz, -CF_2H), 4.52 (2H, s, H-), 2.65 (3H, s, H-10). ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 172.37 (C-8), 162.79 (C-2), 159.75 (C-5), 149.18 (C-4'), 130.75 (C-1'), 130.06 (C-2', C-6'), 126.14 (C-3), 122.54 (C-3', C-5'), 118.38 (C-7), 120.06 (t, J_{FC} = 255 Hz, -$\underline{C}F_2$-CF_2H), 107.49 (t, J_{FC} = 255 Hz, -CF_2-$\underline{C}F_2$H), 46.04 (C-6), 27.18 (C-10). FT-IR (neat): 3184, 1700, 1622, 1383, 1290, 1417 cm^{-1}. HRMS (ESI) (*m/z*) $[M+H]^+$ for ($C_{15}H_{13}N_2O_4F_4$) calculated 361.0811 found 361.0810.

2.1.9. Compound 3i: 1-acetyl-3-(3-(1,1,2,2-tetrafluoroethoxy)benzylidene)piperazine-2,5-dione

Yields: 54%, m.p: 170°C, ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 8.05 (1H, s, NH), 7.51 (1H, t, *J* = 7.8 Hz, H-5'), 7.32 (1H, d, *J* = 7.8 Hz, H-4'), 7.23 (2H, m, H-2', H-6'), 7.13(1H, s, =CH), 6.11 (1H, tt, *J* = 52.8, 2.4 Hz, -CF_2H), 4.51 (2H, s, H-6), 2.65 (3H, s, H-10). ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 172.38 (C-8), 162.74 (C-2), 159.58 (C-5), 149.60 (C-3'), 134.32 (C-1'), 130.91 (C-5'), 126.66 (C-6'), 126.28 (C-7), 122.2 (C-3), 121.76 (C-4'), 118.0 (C-2'), 119.75 (t, J_{FC} = 249 Hz, -$\underline{C}F_2$-CF_2H), 107.49 (t, J_{FC} = 249 Hz, -CF_2-$\underline{C}F_2$H), 46.06 (C-6), 27.21 (C-10). FT-IR (neat): 3191, 1704, 1623, 1427, 1402, 1284 cm^{-1}. HRMS (ESI) (*m/z*) $[M+H]^+$ for ($C_{15}H_{13}N_2O_4F_4$) calculated 361.0811 found 361.0810.

2.1.10. Compound 3j: 1-acetyl-3-(4-ethyl-3-nitrobenzylidene)piperazine-2,5-dione

Yields: 70%, m.p: 200°C, ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 8.74 (1H, s, NH), 7.9 (1H, s, H-2'), 7.56 (1H, d, *J* = 7.9 Hz, H-6'), 7.47 (1H, d, *J* = 7.9 Hz, H-5'), 7.12 (1H, s, =CH), 4.45 (2H, s, H-6), 2.97 (2H, q, *J* = 7.5Hz, CH_2), 2.65 (3H, s, H-10), 1.33 (3H, t, *J* = 7.5, CH_3). ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 172.28 (C-8), 163.66 (C-2), 159.70 (C-5), 149.72 (C-3'), 139.77 (C-4'), 132.96 (C-6'), 132.23 (C-1'), 131.43 (C-5'), 127.08 (C-2'), 124.53 (C-3), 117.30 (C-7), 45.89 (C-6), 27.18 (C-10), 26.02 (CH_2), 14.74 (CH_3). FT-IR (neat): 3197, 1728, 1698, 1522, 1414, 1268 cm^{-1}. HRMS (ESI) (*m/z*) $[M+H]^+$ for ($C_{15}H_{16}N_3O_5$) calculated 318.1090 found 318.1090.

2.1.11. Compound 3k: 1-acetyl-3-(4-butoxy-3-nitrobenzylidene)piperazine-2,5-dione

Yields: 75%, m.p: 166°C, ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 8.86 (1H, s, NH), 7.96 (1H, d, J = 1.9 Hz, H-2'), 7.57 (1H, dd, J = 8.7, 1.9 Hz, H-6'), 7.15 (1H, d, J = 8.7 Hz, H-5'), 7.08 (1H, s, =CH), 4.45 (2H, s, H-6), 4.17 (2H, t, J = 6.7 Hz, CH_2), 2.63 (3H, s, H-10), 1.86 (2H, q, J = 6.7 Hz, CH_2), 1.56 (2H, t, J = 7.2 Hz, CH_2), 1.00 (3H, t, J = 7.2 Hz, CH_3). ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 172.28 (C-8), 163.88 (C-2), 160.02 (C-5), 152.72 (C-4'), 140.02 (C-3'), 134.52 (C-6'), 126.08 (C-5'), 125.80 (C-1'), 124.47 (C-2'), 117.73 (C-3), 115.10 (C-7), 69.71 (CH_2-O), 45.85 (C-6), 30.81 (CH_2), 27.13 (C-10), 19.03 (CH_2), 13.70 (CH_3). FT-IR (neat): 3201, 2991, 1719, 1697, 1518, 1403, 1308 cm^{-1}. HRMS (ESI) (*m/z*) $[M+H]^+$ for ($C_{17}H_{20}N_3O_6$) calculated 362.1352 found 362.1350.

2.1.12. Compound 3l: 1-acetyl-3-(4-chloro-3-nitrobenzylidene)piperazine-2,5-dione

Yields: 56%, m.p: 180°C, ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 9.17 (1H, s, NH), 7.97 (1H, s, H-2'), 7.64 (1H, d, J = 8.2 Hz, H-6'), 7.54 (1H, d, J = 8.2 Hz, H-5'), 7.09 (1H, s, =CH), 4.43 (2H, s, H-6), 2.64 (3H, s, H-10). ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 172.15 (C-8), 164.15 (C-2), 159.44 (C-5), 133.41 (C-3'), 132.73 (C-6'), 132.43 (C-1'), 127.89 (C-5'), 127.33 (C-4'), 125.51 (C-2'), 116.09 (C-7), 45.80 (C-6), 27.18 (C-10). FT-IR (neat): 3286, 1697, 1521, 1397, 1654 cm^{-1}. HRMS (ESI) (*m/z*) $[M+H]^+$ for ($C_{13}H_{11}N_3O_5Cl$) calculated 324.0387 found 324.0392.

2.1.13. Compound 3m: 1-acetyl-3-(2-bromo-5-fluorobenzylidene)piperazine-2,5-dione

Yields: 59%, m.p: 200°C, ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 8.01 (1H, s, NH), 7.67 (1H, dd, J = 8.7, 5.1 Hz, H-3'), 7.12 (1H, s, =CH), 7.09 (2H, m, H-4', H-6'), 4.49 (2H, s, H-6), 2.67 (3H, s, H-10). ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 172.38 (C-8), 163.53 (d, J_{FC} = 248 Hz, C-5'), 162.71 (C-2), 159.03 (C-5), 135.22 (C-1'), 134.38 (C-3'), 127.48 (C-3), 118.68 (C-2'), 118.03 (d, J_{FC} = 22 Hz, C-4'), 117.58 (C-7), 116.40 (d, J_{FC} = 22 Hz, C-6'), 46.13 (C-6), 27.31 (C-10). FT-IR (neat): 3291, 1764, 1705, 1409, 1298 cm^{-1}. HRMS (ESI) (*m/z*) $[M+H]^+$ for ($C_{13}H_{11}N_2O_5BrF$) calculated 340.9937 found 340.9946.

2.1.14. Compound 3n: 1-acetyl-3-(2-bromo-3,4,5-trimethoxybenzylidene)piperazine-2,5-dione

Yields: 30%, m.p: 176°C, ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 7.84 (1H, s, NH), 7.14 (1H, s, =CH), 6.62 (1H, s, H-6'), 4.49 (2H, s, H-6), 3.92 (6H, s, CH_3-O), 3.85 (3H, s, CH_3-O), 2.66 (3H, s, H-10). ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 172.46 (C-8), 162.55 (C-2), 159.26 (C-5), 153.41 (C-3'), 152.07 (C-5'), 143.82 (C-4'), 127.90 (C-1'), 126.60 (C-7), 119.20 (C-3), 111.02 (C-6'), 107.59 (C-2'), 61.21 (CH_3-O), 61.09 (CH_3-O), 56.42 (CH_3-O), 46.22 (C-6), 27.28 (C-10). FT-IR (neat): 3195, 2917, 1702, 1402, 1298, 1258, 1117 cm^{-1}. HRMS (ESI) (*m/z*) $[M+H]^+$ for ($C_{16}H_{18}N_2O_6Br$) calculated 413.0348 found 413.0341.

2.1.15. Compound 3o: 3-((1H-pyrrol-2-yl)methylene)-1-acetylpiperazine-2,5-dione

Yields: 33%, m.p: 204°C, ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 8.94 (1H, s, H-13), 8.25 (1H, s, H-4), 7.07 (1H, s, =CH), 7.03 (H, m, H-17), 6.61 (1H, t, *J* = 2.4 Hz H-15), 6.42 (1H, q, *J* = 2.4 Hz, H-16), 4.52 (2H, s, H-6), 2.62 (3H, s, H-10). ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 172.42 (C-8), 163.45 (C-2), 160.68 (C-5), 125.35 (C-1'), 122.68 (C-3), 120.29 (C-4'), 113.21 (C-2'), 111.96 (C-3'), 111.59 (C-7), 46.06 (C-6), 27.01 (C-10). FT-IR (neat): 3349, 1705, 1619, 1383 cm^{-1}. HRMS (ESI) (*m/z*) $[M+H]^+$ for ($C_{11}H_{12}N_3O_3$) calculated 234.0879 found 234.0878.

2.1.16. Compound 3p: 1-acetyl-3-((3-methylthiophen-2-yl)methylene)piperazine-2,5-dione

Yields: 52%, m.p: 170°C, ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 7.94 (1H, s, NH), 7.45 (1H, d, *J* = 5.1 Hz, H-5'), 7.38 (1H, s, =CH), 7.01 (1H, d, *J* = 5.1 Hz, H-4'), 4.52 (2H, s, H-6), 2.64 (3H, s, H-10), 2.38 (3H, s, CH_3). ^{13}C-NMR (75 MHz, $CDCl_3$) δ ppm: 172.35 (C-8), 162.75 (C-2), 160.53 (C-5), 142.05 (C-3'), 131.23 (C-5'), 128.96 (C-4'), 127.20 (C-2'), 122.50 (C-3), 112.29 (C-7), 45.98 (C-6), 27.01 (C-10), 14.54 (CH_3). FT-IR (neat): 3261, 1709, 1621, 1401, 1273 cm^{-1}. HRMS (ESI) (*m/z*) $[M+H]^+$ for ($C_{12}H_{13}N_2O_3S$) calculated 265.0647 found 265.0651.

2.2. Biological assays

2.2.1. Anti-acetylcholinesterase activity

The acetyl-cholinesterase inhibitory activity was performed by Ellman's method as previously described [12]. 25 µL of each compound (100 µM) and 50 µL of 100 mM sodium phosphate buffer solution (pH = 8), 125 µL of 3 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) solution prepared in 100 mM phosphate Buffer solution (pH = 7) and 25 µL of the enzyme AChE solution (1.4 U/mL) dissolved in 20 mM phosphate buffer solution (pH = 7) were added in a 96-well micro-plate and incubated at 25 °C for 15 min. Then, 25 µL of 15 mM acetylthiocholine iodide solution prepared in 100 mM sodium phosphate buffer (pH = 8) was added to the mixture. The final mixture was incubated for 10 min at 25 °C and the absorbance was measured at 412 nm against a blank without compound. Galanthamine was used as a positive control.

2.2.2. Anti-xanthine oxidase activity

The xanthine oxidase inhibitory activity of diketopiperazine derivatives (**1** and **3a-p**) was determined using the spectrophotometric measurement as previously reported [12]. Briefly, 60 µL of 70 nM phosphate buffer solution (pH = 7.5), 50 µL of each compounds (100 µM),

30 µL of 0.1 U/mL xanthine enzyme solution were added in a 96-well micro-plate and incubated for 15 min at 25°C. The plate was recuperated and 60 µL of 150 µM xanthine solution were added and the final mixture was then incubated for 5 min. The absorbance was read at 295 nm against a blank without the compounds. Allopurinol was used as a positive control.

2.2.3. Anti-diabetic activity

The anti-diabetic assay was carried out by a modified procedure of Shalaby et al. [13] using α-amylase enzyme. Briefly, 50 µL of each compound (100 µM per well), 50 µL of 1 U/mL α-amylase enzyme prepared in sodium phosphate buffer solution (pH = 6.9) were placed in a tube and pre-incubated for 15 min at 25°C. The tube was recuperated and 100 µL of 1% starch solution prepared in sodium buffer solution (pH = 6.9) was added. The mixture was then pre-incubation for 5 min at 25°C, after which 100 µL of dinitrosalicylic acid (DNS) reagent was added and then further incubated in boiling water for 10 min. The mixture was finally diluted with 1 mL sodium phosphate buffer solution (pH = 6.9) and the absorbance was read at 540 nm against a blank without the compound. Acarbose was used as positive control.

2.2.4. Cytotoxic activity

Cytotoxicity of compounds was estimated on three human cell lines; colon (HCT-116), human breast (MCF-7) and ovarian (OVCAR-3) cancer cells as reported by Kammoun El Euch et al., [12]. 100 µL of cells, at a density of 10^4 cells/well, were seeded in 96-well plates and maintained for 24 h at 37°C. Then, 100 µL of culture medium containing various compound or control were added. After 48 h incubation, the medium was removed and cells were treated with 50 µL of 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution, prepared in phosphate buffered saline (PBS), at a concentration of 1 mg/mL and then further incubated for 40 min at 37°C. Finally, MTT solution was absorbed and 50 µL of DMSO were added to dissolve insoluble dark blue formazan crystals and absorbance was measured at 605 nm using spectrophotometer. Tamoxifen was used as positive control.

Data analysis and statistics

Data were reported as means ± standard deviation (S.D.) of three replicates and analyzed using analysis of variance (ANOVA), and means were compared by the test of least significant difference (LSD) at P=0.05 using XLSTAT for Windows.

3. Results and discussion

3.1. Synthesis

The treatment, at room temperature for about 3 hours, of 1,4-diacetylpiperazine-2,5-dione **1** (1.5 equiv.) with a series of arylaldehydes (1 equiv.) in the presence of Cs_2CO_3 (1.5 equiv.) as a base in dry *N,N*-dimethylformamide provided exclusively the 1-acetyl-3-arylidenepiperazine-2,5-diones 3a-p, as illustrated in scheme 1 [2]. Further, ^{1}H NMR spectra (300 MHz, $CDCl_3$) of compounds **3a-p** displayed, in addition of the protons introduced by 1,4-diacetylpiperazine-2,5-dione **1**, a characteristic singlet at δ_H 7.07-7.38 attributable to the ethylenic proton, H-7 of the arylidene moiety formed and signals (AA'BB' spin patterns in the case of compounds **3a-h**) at δ_H 6.11-7.97 easily attributable to the aromatic protons introduced by the arylaldehyde used. Furthermore, the same spectra allowed to note the disappearance of an acetyl group by the observation of the reduction of the integration of the corresponding signal which passes from 6H to 3H at δ_H 2.62-2.67 Indeed, the deacetylation of the nitrogen in the 4 position was deduced from the HMBC spectrum showing the correlation of the methylene protons H-6 (δ_H 4.43-4.52) with the three carbonyl functions C-2 (δ_C 164.15-162.66), C-5 (δ_C 160.41-159.03) and C-8 (δ_C 172.50-172.15). All chemical shifts and multiplicities were in good agreement with the proposed structures.

1 → Ar–CHO **2**, Cs_2CO_3 / DMF → **3a-p**

3a R= 4-CH_3O.Ph
3b R= 4-CH_3S.Ph
3c R= 4-Cl.Ph
3d R= 4-CF_3.Ph
3e R= 4-morpholino.Ph
3f R= 4-phenoxy.Ph
3g R= 4-benzyloxy.Ph
3h R= 4-C_2HF_4O.Ph
3i R= 3-C_2HF_4O.Ph
3j R= 4-C_2H_5-3NO_2.Ph
3k R= 4-C_4H_9O-3-NO_2.Ph
3l R= 4-Cl-3-NO_2.Ph
3m R= 2-Br-5-F.Ph
3n R= 2-Br-3,4,5-CH_3O.Ph
3o R= pyrrolyl
3p R= 3-methylthienyl

Scheme 1. Synthesis of arylidene 2,5-diketopiperazine derivatives **3a-p**.

The ^{13}C-NMR spectra confirmed the above ^{1}H NMR data, by particularly the observation of a new sp^2 methine group C-7 at δ_C 112.29-126.60 and signals at δ_C 160.32-107.59 relative to carbons of the aromatic systems in addition to the carbon signals introduced by the 2,5-diketopiperazine **1** skeleton. The HRMS mass spectra were in agreement with the molecular formula of all the synthesized compounds.

We should notice that although in some cases, the yield was limited, the reaction made resulted in a single product, thus showing its high regioselectivity. All the prepared compounds are new except derivatives **3a** and **3f** which have been reported in previous works [14,15]. All the synthesized compounds can be easily dissolved in normal solvents, such as chloroform, dichloromethane (DCM), ethyl acetate (AcOEt), N,N-dimethylmethanamide (DMF), dimethylsulfoxide (DMSO), methanol (MeOH) and ethanol (EtOH).

3.2. Biological activities

3.2.1. Anti-acetylcholinesterase activity

Table 1. Acetylcholinesterase, xanthine oxidase and α-amylase inhibition capacities of compound 1 and 2,5-diketopiperazine derivatives 3a-p.

Compd.	Anti-acetylcholinesterase	Anti-xanthine oxidase	Anti-α-amylase
	% of Inhibition at 100 µM		
1	3.9 ± 0.6^{g}	na	8.7 ± 0.6^{h}
3a	6.5 ± 0.5^{f}	21.0 ± 0.5^{d}	12.4 ± 0.4^{g}
3b	11.9 ± 1.9^{e}	na	22.8 ± 0.1^{e}
3c	na	6.5 ± 0.7^{f}	23.0 ± 0.2^{e}
3d	15.4 ± 2.2^{d}	11.3 ± 0.1^{e}	56.7 ± 1.2^{a}
3e	13.2 ± 2.2^{e}	na	19.8 ± 0.6^{e}
3f	na	3.8 ± 0.7^{g}	57.8 ± 1.9^{a}
3g	na	$2.9\pm0.7^{g,h}$	27.8 ± 0.2^{d}
3h	na	11.0 ± 1.0^{e}	20.3 ± 1.7^{e}
3i	6.7 ± 0.9^{f}	4.3 ± 0.2^{g}	5.8 ± 1.0^{h}
3j	15.9 ± 1.3^{d}	44.3 ± 3.8^{c}	$14.8\pm2.0^{f,g}$
3k	32.6 ± 0.8^{b}	58.7 ± 3.2^{a}	30.9 ± 0.8^{c}
3l	4.7 ± 2.2^{f}	1.4 ± 0.6^{h}	50.3 ± 2.5^{b}
3m	na	na	$14.2\pm1.8^{f,g}$

3n	na	na	na
3o	na	na	14.5±2.0[f,g]
3p	29.9±0.5[c]	43.6±2.9[c]	15.0±1.1[f]
Galanthamine (4 µM)	49.0±1.2[a]	-	-
Allopurinol (8µM)	-	48.8±0.8[b]	-
Acarbose (200 µM)	-	-	53.1±3.0[b]

Means with the same letters in a column are not significantly different at $P<0.05$.

The anti-AChE inhibitory potential of the starting material **1** and the newly synthesized 2,5-diketopiperazines **3a-p** was screened at the concentration of 100 µM by considering percentage of inhibition (PI %) as described by Kammoun El Euch et al. [12]. The Results given in Table 1 showed that the most of our compounds exhibited weak to moderate anti-AChE activity.

Values of percentage of inhibition ranging from 3.9±0.6 to 32.6±0.8% were obtained. Our finding showed that compounds **3k** (4-C_4H_9O-3-NO_2.Ph) and **3p** with a 3-CH_3-thiophene system displayed the relatively highest anti-AChE effect (PI% = 32.6±0.8 and 29.9±0.5%, respectively). On the other hand, the relatively high activity of the derivative **3k** compared to those of its analogues **3j** and **3l** nitrated at the same position (C-3), clearly shows the contribution of the butoxy fragment to this activity compared to the ethyl group in **3j** (PI = 15.9±1.3%) and the chlorine atom in **3l** (PI = 4.7±2.2%). Moreover, the inactivity of the derivative **3c** (4-Cl.Ph) compared to its analogue **3l** (4-Cl-3-NO_2.Ph) slightly active, could on the other hand show also the contribution of the nitro group to the anti-AChE activity of the compound **3k** (4-C_4H_9O-3-NO_2.Ph).

3.2.2. Anti-xanthine oxidase activity

The compound **1** and all synthesised compounds **3a-p** were screened for their XOD inhibitory potential as described in the literature [12]. The percentage of inhibition was determined for all compounds (**1** and **3a-p**), tested at the concentration of 100µM, and the results are given in Table 1. Our findings showed that all synthesized compounds exhibited weak to moderate anti-XOD activity. Only **3k** (4-C_4H_9O-3-NO_2.Ph), **3j** (4-C_2H_5-3-NO_2.Ph) and **3p** (3-CH_3-thiophene) were found to be active with percentage inhibition values of 58.7±3.2, 44.3±3.8 and 43.6±2.9%, respectively compared to the rest of the tested compounds including the starting material **1** which showed no activity. The relatively high activity of the derivative **3k** (4-C_4H_9O-3-NO_2.Ph) compared to that of its analogue **3j** (4-C_2H_5-3-NO_2.Ph) can be explained by the largest donor effect of the butoxy fragment (+M) compared to that produced by the

ethyl group (+I). On the other hand, although weak, the relatively high activity of the derivative **3c** (4-Cl.Ph) compared to its analogue **3l** (4-Cl-3-NO_2.Ph) shows that the NO_2 group in position 3' is not in favor of this activity, therefore, this group in **3j** and **3k** did not seem to contribute to their anti-XOD activity. The activity of compound **3p** (PI = 43.6±2.9%) is certainly due to the particular structure of the 3-CH_3-thiophene moiety compared to the rest of the aryl systems introduced by the arylaldehydes used. It has also been found that the compound **3a** with a 4-CH_3O.Ph group exhibited an anti-XOD activity with a percentage inhibition value of 21.0±0.5% whereas its analogue **3b** with a 4-CH_3S.Ph system did not show any activity. This finding clearly shows that the nature of the heteroatom was at the origin of this difference. All the halogenated derivatives did not display any activity against xanthine oxidase enzyme. These results demonstrated the effect of the halogen atoms (F, Cl and Br) directly attached to the phenyl ring or through a methylene group to lose the anti-OXD activity.

3.2.3. Anti-diabetic activity

The anti-diabetic activity using α-amylase inhibition method as described by Shalaby et al. [13] with a slight modifications and the observed percentage of inhibition are presented in Table 1. The anti-diabetic screening data revealed that all the synthesised compounds showed weak to moderate inhibitory activity with percentage values ranging from 5.8±1.0 and 57.8±1.9 % expect compound **3n** with 2-bromo-3,4,5-trimethoxyphenyl moiety which did not exhibit any inactive.

The higher inhibitory activity was displayed by compound **3f** with a 4-phenoxyphenyl moiety attached to the protected diketopiperazine (PI% = 57.8±1.9%). Moreover, the derivatives **3d** (4-CF_3.Ph) and **3l** (4-Cl-3-NO_2.Ph) showed considerable activities against α-amylase (PI = 56.7±1.2 and 50.3±2.5%, respectively). The relatively high activity of compound **3f** (4-PhO.Ph; PI= 57.8±1.9%) compared to those of its analogues **3a** (4-CH_3O.Ph; PI = 12.4±0.4%) and **3b** (4-CH_3S.Ph; PI = 22.8±0.1%) suggests that in addition to the nature of the substituent attached to the phenyl moiety, the longer the conjugation is extended more the better activity.

On the other hand, it was found that compound **3l** (4-Cl-3-NO_2.Ph) is almost twice more active than its analogue **3c** (4-Cl.Ph). This finding showed the contribution of the NO_2 group to the activity of **3l** and probably largely explains the activity of compound **3k** (4-C_4H_9O-3-NO_2.Ph; PI = 30.9±0.8%) if we consider that the alkyloxy group is not a major factor bearing activity against α-amylase such as the case of **3a** (4-CH_3O.Ph; PI = 12.4±0.4%). It is also interesting to note the activity three times higher of compound **3h** (4-CHF_2CF_2O.Ph; PI =

20.3±1.7%) where the 1,1,2,2-tetrafluoroethoxy group is attached at the 4 position of the aromatic ring compared to its positional isomer **3i** (PI = 5.8±1.0%) where this group is in the 3 position.

3.2.4. Cytotoxic activity

For evaluation of cytotoxic activity of compound **1** and the new synthesized piperazine derivatives **3a-p**, three human cancer cell lines were used: HCT-116 (colon carcinoma), MCF-7 (breast carcinoma) and OVCAR (ovarian cancer) and the observed percentage of inhibition at 100 µM given in Table 2 showed that most compounds exhibited moderated to good cytotoxic effects. The results showed that the starting material **1** exhibited a moderate cytotoxic activity against OVCAR-3 (PI = 41.3±2.9%) but HCT-116 and MCF-7 cell lines do not seem enough sensitive to this compound (PI = 26.5±2.1 and 20.7±3.0%). Among the piperazines prepared compound **3g** (4-$PhCH_2O$.Ph) was found the most cytotoxic against HCT-116, MCF-7 and OVCAR-3 cell lines used (PI= 83.2±2.4, 89.6±4.9 and 74.4±5.2%, respectively). This activity compared to that of **3f** (PI = 62.2±4.8, 76.0±6.3 and 64.7±1.5%, respectively) shows the importance of the methylene group placed between the phenyl and the oxygen atom in the structure of **3g** limiting the extended conjugation which probably was the cause of the attenuation of the activity of compound **3f**. The piperazine derivative **3b** (4-CH_3S.Ph) was found to be more active than its analogue **3a** (4-CH_3O.Ph) towards the HCT-116, MCF-7 and OVCAR-3 cell lines used (PI = 52.6±4.9-61.6±2.8 and 20.7±3.0-41.3±2.9%, respectively). This finding clearly shows the importance of the nature of the heteroatom in this activity, in fact, the sulfur atom seems to bring more activity than the oxygen atom.

On the other hand, the relatively high cytotoxic activity of the derivative **3l** (4-Cl-3-NO_2.Ph) towards the three cancer cell lines (HCT-116 (PI = 56.8±4.0%), MCF-7 (PI = 57.7±7.3%) and OVCAR-3 (PI = 69.4±3.1%)) compared to its analogue **3c** (4-Cl.Ph) (PI = 23.0±3.7, 34.9±1.3 and 34.3±4.1%, respectively) shows that the NO_2 group in position 3' is in favor of this activity, therefore, this group in **3k** (4-C_4H_9O-3-NO_2.Ph) seems to contribute to its activity against the three cell lines used (PI= 64.3±0.8, 65.5±3.7 and 64.9±0.5%, respectively) but in **3j** (4-C_2H_5-3-NO_2.Ph) it appears effective only against MCF-7 (PI = 58.4±8.0%) and OVCAR-3 (PI = 71.4±0.9%). This finding also showed the selectivity of the cell lines used towards the tested compounds. The results showed that the fluorinated compounds **3d** (4-CF_3.Ph), **3h** (4-CHF_2CF_2O.Ph), **3i** (3-CHF_2CF_2O.Ph) and **3m** (2-Br-5-F.Ph) were found relatively more active against OVCAR-3, hence the selectivity even of the cells used to these compounds. Indeed, the derivative **3m** showed the highest activity (PI = 77.0±2.1%) followed

by **3d** (PI = 59.7±3.3%). The activity of compound **3m** may also due to the coexistence of the two halogens (F and Br). This eventuality could be probably supported by the percentage of inhibition shown by the brominated derivative **3n** (PI = 56.9±6.7%) against the same cell line. Comparable activities of the fluorinated positional isomers **3h** and **3i** do not show that the position (3 or 4) of the fluorinated fragment in the aromatic ring greatly influences the activity. However, the 4-position appears slightly in favor of the activity against OVCAR-3 (PI = 49.7±3.7 and 42.2±2.4%, respectively).

Table 2. Cytotoxic activity (HCT-116, MCF-7 and OVCAR-3) of compound 1 and 2,5-diketopiperazine derivatives **3a-p**.

Compd.	**HCT-116**	**MCF-7**	**OVCAR-3**
	Percent of inhibition (%) at 100 µM		
1	26.5±2.1[h,i]	20.7±3.0[j]	41.3±2.9[k]
3a	29.1±1.2[g,h]	31.1±3.9[i]	44.2±3.8[j,k]
3b	52.6±4.9[e]	57.4±4.6[e]	61.6±2.8[g]
3c	23.0±3.7[i,j]	34.9±1.3[h,i]	34.3±4.1[l]
3d	38.2±5.1[f]	37.7±1.8[g]	59.7±3.3[g,h]
3e	20.6±2.4[j,k]	46.0±3.2[f]	56.0±3.5[h]
3f	62.2±4.8[c]	76.0±6.3[c]	64.7±1.5[f]
3g	83.2±2.4[b]	89.6±4.9[b]	74.4±5.2[b,c]
3h	39.2±6.7[f]	31.1±2.8[i]	49.7±3.7[i]
3i	40.2±1.4[f]	36.5±3.3[g,h]	42.2±2.4[j,k]
3j	33.4±1.8[g]	58.4±8.0[e]	71.4±0.9[c,d]
3k	64.3±0.8[c]	65.5±3.7[d]	64.9±0.5[f]
3l	56.8±4.0[d]	57.7±7.3[e]	69.4±3.1[d,e]
3m	64.4±0.6[c]	60.6±5.3[e]	77.0±2.1[b]
3n	9.9±1.9[l]	46.2±1.3[f]	56.9±6.7[h]
3o	16.1±1.3[k]	21.8±0.8[j]	45.6±0.5[i,j]
3p	51.7±3.7[e]	58.2±7.1[e]	65.9±4.4[e,f]
Tamoxifen (5 µM)	100.7±4.7[a]	98.5±5.6[a]	98.8±4.9[a]

Means with the same letters in a column are not significantly different at $P<0.05$.

CONCLUSION

In summary, we have described herein the successful access to sixteen novel arylidene diketopiperazines **3a-p** *via* Claisen-Schmidt condensation of the *N,N*-diacetyl-diketopiperazine **1** with a series of substituted arylaldehydes. The reaction was conducted with a high regioselectivity. All compounds have been characterized by their full spectral data and screened for their anti-acetylcholinesterase, anti-xanthine oxidase, anti-diabetic and anticancer (HCT-116, MCF-7 and OVCAR-3) activities.

The results showed that most of the tested compounds showed limited anti-acetylcholinesterase activity, and some compounds displayed a moderate anti-diabetic activity which depended on the nature of the substituent attached to the aromatic group introduced by the aldehyde used. By cons, most piperazines 3a-p were considered moderate to good cytotoxic agents. The derivative 3g (4-$PhCH_2O$.Ph) was found to be the most cytotoxic against HCT-116, MCF-7 and OVCAR-3 cell lines (PI= 83.2±2.4, 89.6±4.9 and 74.4±5.2%, respectively) followed by 3m (2-Br-5-F.Ph) then 3j (4-C_2H_5-3-NO_2.Ph) both exhibited good cytotoxicity against OVCAR-3 (PI= 77.0±2.1 and 71.4±0.9%, respectively). Our finding also allowed to note the selectivity of the cell lines used towards most tested compounds.

REFERENCES

[1] McCleland K, Milne PJ, Lucieto FR, Frost C, Brauns SC, Van De Venter M, Du Plessis J, Dyason K. An investigation into the biological activity of the selected histidine-containing diketopiperazines cyclo(His-Phe) and cyclo(His-Tyr). *J. Pharm. Pharmacol.* **2004**, 56, 1143-1153.

[2] Martins MB, Carvalho I. Diketopiperazines: Biological activity and synthesis. *Tetrahedron.* **2007**, 63, 9923-9932.

[3] Folkes A, Roe MB, Sohal S, Golec J, Faint Rc, Brooks T, Charlton P. Synthesis and *in vitro* evaluation of a series of diketopiperazine inhibitors of plasminogen activator inhibitor-1. *Bioorg. Med. Chem. Lett.* **2001**, 11, 2589-2592.

[4] Nicholson B, Lloyd GK, Miller BR, Palladino MA, Kiso Y, Hayashi Y, Neuteboom STC. NPI-2358 is a tubulin-depolymerizing agent: in-vitro evidence for activity as a tumor vascular-disrupting agent. *Anti-Cancer Drugs* **2006**, 17, 25-31.

[5] Sinha S, Srivastava R, De Clercq E, Singh RK. Synthesis and antiviral properties of arabino and ribonucleosides of 1,3-dideazaadenine, 4-nitro-1,3-dideazapurine and diketopiperazine. *Nucleosides Nucleotides Nucleic Acids.* **2004**, 23, 1815-1824.

[6] Byun HG, Zhang H, Mochizuki M, Adachi K, Shizuri Y, Lee WJ, Kim SK. Novel antifungal diketopiperazine from marine fungus. *J. Antibiot*. **2003**, 56, 102-106.

[7] Fdhila F, Vazquez V, Sanchez JL, Riguera R. dd-diketopiperazines: antibiotics active against *Vibrio anguillarum* isolated from marine bacteria associated with cultures of *Pecten maximus*. *J. Nat. Prod.* **2003**, 66, 1299-1301.

[8] Borthwick AD. 2,5-Diketopiperazines: synthesis, reactions, medicinal chemistry, and bioactive natural products. *Chem. Rev.* **2012**, 112, 3641-3716.

[9] Loughlin WA, Marshall RL, Carreiro A, Elson KE. Solution-phase combinatorial synthesis and evaluation of piperazine-2,5-dione derivatives. *Bioorg. Med. Chem. Lett.* **2000**, 10, 91-94.

[10] Ando S, Grote AL, Koide K. Diastereoselective synthesis of diketopiperazine bis-alpha, beta-epoxides. *J. Org. Chem.* **2011**, 76, 1155-1158.

[11] Weatherhead-Kloster RA, Selby HD, Miller WB, Mash EA. Organic crystal engineering with 1,4-piperazine-2,5-diones. 6. Studies of the hydrogen-bond association of cyclo[(2-methylamino-4,7-dimethoxyindan-2-carboxylic acid) (2-amino-4,7-dimethoxyindan-2-carboxylic acid)]. *J. Org. Chem.* **2005**, 70, 8693-8702.

[12] Kammoun El Euch S, Bouajila J, Bouzouita N. Chemical composition, biological and cytotoxic activities of *Cistus salviifolius* flower buds and leaves extracts. *Ind. Crops Prod.* **2015**, 76, 1100-1105.

[13] Shalaby NMM, Abd-Alla HI, Aly HF, Albalawy MA, Shaker KH, Bouajila J. Preliminary *in vitro* and *in vivo* evaluation of antidiabetic activity of *Ducrosiaanethifolia Boiss* and its linear furano coumarins. *BioMed. Res. Int. ID 480545.* **2014**, 1-13.

[14] Juan GF, de la Guesta GE, Avendaño C. Atom-efficient synthesis of 2,6-diazacyclophane compounds through alcoholysis/reduction of 3-nitroarylmethylene-2,5-piperazinediones. *Tetrahedron* **2008**, 64, 2762-2771.

[15] Adriano M, Roberto C, Serena F, Salvatore G, Azzurra S, Veronique M, Robert K, Francesco E. Synthesis and anti-cancer activity of naturally occurring 2,5-diketopiperazines. *Fitoterapia* **2015**, 98, 91-97.

Conclusion

Les résultats ont montré que tous les composés synthétisés peuvent être facilement dissous dans des solvants ordinaires tels que le chloroforme, le dichlorométhane (DCM), l'acétate d'éthyle (AcOEt), le N, N-dimethylmethanamide (DMF), le diméthylsulfoxyde (DMSO), le methanol (MeOH) et l'éthanol (EtOH). Les analogues structuraux de la 2,5-dicétopipérazine ont été testés pour leurs activités anti-acétylcholinestérase, anti-diabétique, anti-xanthine oxydase et cytotoxique. Les résultats ont montré que la plupart des composés testés ont montré une activité anti-acétylcholinestérase limitée, et certains composés ont montré une activité anti-diabétique modérée qui dépend de la nature du substituant fixé au groupe aromatique introduit par l'aldéhyde utilisé. Par contre, la plupart des pipérazines synthétisés ont montré une moyenne à forte toxicité contre les lignées cancéreuses à 100 µM (HCT-116, MCF-7 et OVCAR-3). Le dérivé 3g (4-PhCH2O.Ph) a montré une forte toxicité contre les lignées cellulaires (HCT-116, MCF-7 et OVCAR-3) (PI = 83,2 ± 2,4, 89,6 ± 4,9 et 74,4 ± 5,2%, respectivement), de plus les dérivés 3m (2-Br-5-F.Ph) et 3j (4-C2H5-3-NO2.Ph) ont montré une bonne cytotoxicité contre OVCAR-3 (PI = 77,0 ± 2,1 et 71,4 ± 0,9%, respectivement).

Conclusion générale

Les travaux de recherche objet de cette thèse s'inscrivent dans le cadre de la contribution à l'étude et l'identification des métabolites secondaires à partir des microorganismes. Nous nous sommes intéressés aux aspects microbiologiques, chimiques, spectroscopiques, biologiques et agronomiques de deux souches bactériennes ; *B. megaterium* et *Burkholderia* sp.. Trois parties ont été développées :

• Les travaux de recherche ont commencé par l'étude de l'influence des conditions de culture telle que la source de carbone, le temps d'incubation ainsi que l'interaction bactérienne sur la sécrétion des composés volatiles bactériens. Pour se faire, nous avons développés plusieurs études qui sont :

- Des études sur l'identification des différents volatiles secrétés à partir de *Burkholderia* sp. en utilisant deux sources de carbone différentes. Les résultats obtenus laissent penser fortement que les sources de carbone influence directement la production des volatiles bactériens.

- Les invistigations chimiques entreprises sur les deux bactéries ainsi que leur mélange nous ont permis d'identifier plus que cent composés volatils dont certains identifés pour la première fois à partir des bactéries (*N*-butylbenzenesulfonamide, triacontane, octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate (*E*)-5-chloro-3-(hydroxyimino)indolin-2-one, *N*,*N*-diethyl-10,13-dimethyl-17-(6-methylheptan-2-yl)-4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-amine et 6-((*E*)-4-((*E*)-2-(5-hydroxy-2-methylenecyclohexylidene)ethylidene)-7a-methyl-octahydro-1*H*-inden-1-yl)-2-methylheptane-2,3-diol).

- Des études analytiques qui se basent sur l'analyse de la composition des métabolites volatiles lors d'une interaction bactérienne. Durant ces analyses nous nous sommes intéressés d'une part à l'analyse des composés volatiles émis par *B. megaterium* et *Burkholderia* sp. cultivés séparément, et d'autre part à l'analyse des composés volatiles émis d'un mélange contenant à la fois *B. megaterium* et *Burkholderia* sp.. Nous avons constaté que les interactions bactériennes influencent énormément la production des composés volatils à partir de deux souches étudiées.

- Les dernières études se sont intéressées à l'impact de stockage sur la production des composés volatiles à partir de *B. megaterium*, *Burkholderia* sp. et le mélange contenant les deux souches. Ces études ont permis de renforcer de plus en plus que la production des composés volatiles bactériens qui sont influencés et directement connectés aux conditions de culture des bactéries.

• Les analyses des composés volatils précités ont été consolidées par une évaluation de quelques activités biologiques. Dans ce cadre, nous nous sommes intéressés à l'étude des activités anti-acétylcholinestérase, anti-5-lipoxygénase, anti-xanthine oxydase, anti-α-amylase, cytotoxique ainsi que les potentiels allélopathique des différents extraits préparés de différentes souches bactériennes. Les résultats obtenus peuvent être résumés comme suit :

- La substitution de dextrose par glycérol a affecté les activités biologiques en augmentant les valeurs des activités anti-α-amylase, anti-acétylcholinestérase et cytotoxique, contrairement à l'activité anti-5-lipoxygénase et le potentiel allélopathique qui diminuent.
- Les interactions bactériennes, résultant de la combinaison de *B. megaterium* avec *Burkholderia* sp., ont affecté les résultats des différents activités biologiques en améliorant surtout les valeurs des activités anti-α-amylase et anti-5-lipoxygénase.
- Le stockage à longue durée des différentes souches a affecté les résultats de différentes activités biologiques testées.

Suite aux résultats précités, nous avons également démontré la présence d'une corrélation entre les conditions de culture de deux souches étudiées et la production des composés volatils bactériens ainsi que les activités biologiques.

• l'importance biologique et pharmacologique des dicétopperazines, telle que les composés que nous avons identifié au cours de ce travail ou celle identifié d'autre souche bactérienne nous a incité à synthétiser une série d'analogues structuraux. Ainsi, , notre intérêt s'est porté vers la synthèse des nouveaux dérivés protégés de dicétopipérazine **3a-p** ainsi que l'étude de quelques activités biologiques de différent produits synthétisés.

Ar–CHO, Cs_2CO_3 / DMF → **3a-p**

La majorité des molécules préparées dans le cadre de ce travail sont nouvelles. Les résultats biologiques obtenus des produits synthétisés ont montré qu'un certain nombre d'analogues de DKP sont dotés des activités anti-α-amylase et cytotoxiques intéressantes.

Perspectives

Dans la suite de ces travaux, il sera intéressant de poursuivre l'étude chromatographique des extraits les plus actifs, dont l'objectif est d'isoler de nouveaux produits naturels biologiquement actifs. L'étape de l'identification des constituants des extraits ayant des bonnes activités par LC-HRMS est aussi intéresante.

D'autre part, des études *in vivo* (en collaboration), en fonction des applications, sont à prévoir pour les extraits, les moélcules isolées ou les composés synthétisés les plus actifs.

Nous continuerons à élargir et à modifier d'autres facteurs pour bien comprendre les rôles des composés volatils microbiens ainsi que pour améliorer les résultats des tests biologiques et pharmacologiques des souches bactériennes.

Nous visons aussi continuer la synthèse de nouveaux dérivés dicétopipérazines pour améliorer et valoriser les activités antidiabétiques et cytotoxiques en exploitant par exemple l'autre fonction amine protégé. Nous pouvons penser aussi à l'allylation et à la propargylation du groupe OH pour accéder, via la réaction de cycloaddition, à de nouveaux aziridines et triazoles.

Résumé
Au cours de ce travail de thèse intitulé « Identification chimique de métabolites secondaires de certains microorganismes, évaluation de leurs effets dans les domaines pharmaceutiques et agronomiques », nous nous sommes intéressés à l'étude des effets des conditions de culture sur la production des composés organiques volatils microbiens à partir de deux souches bactériennes co-existantes dans le sol Français : *Burkholderia* sp. et *Bacillus megaterium.*
A partir des différents extraits préparés, plus que cent composés ont été identifiés, comprenant les dicétopipérazines, les alcools, les composés soufrés, les esters et les acides carboxyliques, par le biais de plusieurs techniques chimiques, analytiques et spectroscopiques. Les résultats obtenus ont montré que les conditions de culture sont les pricipales responsables de la production des différentes familles chimiques des volatiles. Nous avons identifiés des composés qui sont rapportés pour la première fois à partir des bactéries tel que: la *N*-butylbenzènesulfonamide, triacontane, le proponaoate de 3- (3,5-di-tert-butyl-4-hydroxyphényl), (*E*) -5-chloro-3-(hydroxyimino) indoline-2-one et 1,3,5-triméthyl-2-octadecylcyclohexane.
Sur le plan biologique, on a montré que les résultats obtenus sont fortement influencés par les conditions de culture utilisées pour cultiver les bactéries testées. En parallèle à cette investigation, nous avons montré que les extraits de *Burkholderia* sp. sont dotés d'un très important potentiel allélopathique.
Enfin, une série des analogues de dicétopipérazines a été préparée et évaluée pour leurs activités anti-xanthine oxydase, anti α-amylase et anti 5-lipoxygénase ainsi que pour leurs activités cytotoxiques contre les lignées cellulaires suivantes ; OVCAR, MCF7 et HCT116. Un certain nombre de ces dérivés de dicétopiperazine ont montré des activités anti α-amylase et cytotoxique importantes.
Mots clés : *Burkholderia* sp., *Bacillus megaterium*, GC-HRMS, derivatisation reactions, dicétopiperazine, anti-xanthine oxidase, anti α-amylase, anti 5-lipoxygenase, cytotoxique, potentiel allélopathique.
Abstract
In this thesis entitled « Chemical identification of secondary metabolites from microorganism, evaluation of their effects on pharmaceutical and agronomic fields », we are interested in studying the effect of culture conditions on the production of microbial volatiles organic compounds by two bacteria that inhabit French soil which are: *Burkholderia* sp. and *Bacillus megaterium.* From different prepared extracts, more than one hudred compounds were identified, including diketopiperazine, alcohols, sulfur containing compounds, esters and carboxylic acids, by means of several chemical, analytical and spectroscopic techniques. Results showed that culture conditions of different bacteria are the mainly responsible of production of different blend of volatiles.Many identified compounds including *N*-butylbenzenesulfonamide, triacontane, octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propanoate, (*E*)-5-chloro-3-(hydroxyimino)indolin-2-one and 1,3,5-trimethyl-2-octadecylcyclohexane are reported for the first time from bacteria.
Biologically, we have shown that obtained results are greatly influenced by the cultures conditions used in cultivation of tested bacteria. In addition to that, we have shown that *Burkholderia* sp. extracts possessed a very good allelopathic potential.
Finally, a series new protested deketopiperazine derivatives have been prepared and evaluated *in vitro* against xanthine oxidase, α-amylase and 5-lipoxygenase enzymes, OVCAR, MCF7 and HCT116 cancer cell lines. Some of these molecules have been shown to be potent inhibitors of α-amylase and different cancer cell lines.
Keywords: *Burkholderia* sp., *Bacillus megaterium*, GC-HRMS, derivatisation reactions, diketopiperazine, anti-xanthine oxidase, anti α-amylase, anti 5-lipoxygenase, cytotoxic, allelopatic potential.